Vol. 116, No. 2 April, 1959

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

TWO NEW GENERA OF DINOFLAGELLATES FROM CALIFORNIA[1]

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The coastal waters in the San Diego region support fairly abundant populations of phytoplankton. Armored dinoflagellates of this region were studied extensively by Kofoid and his associates (1907–1933), but there are still numerous undescribed or little known representatives especially among the smaller species. In the present paper, two new genera and species are described. These were originally isolated by Dr. Beatrice M. Sweeney in 1956–57 from coastal water at La Jolla, Calif., and have since been maintained as laboratory cultures.

Acknowledgment is here made to Prof. Francis T. Haxo and Prof. Martin W. Johnson for their interest and for providing research facilities. The author is indebted to Dr. Beatrice M. Sweeney whose cultures made this study possible, to Mrs. Anne Dodson for valued technical aid and to Dr. K. A. Clendenning for assistance in the preparation of the manuscript.

Methods

The dinoflagellates were first examined alive. Fixed material was then studied under an oil immersion objective and by phase contrast. To derive the general plate formulae of the thecae, an individual cell was isolated under a cover-glass. A drop of concentrated sodium hypochlorite solution was then passed slowly under the cover-glass to destroy the protoplasm and to remove the cement which unites the plates. This process was assisted by applying very gentle pressure to the cover-glass, but great caution was necessary because of the fragility of the specimens. With *Scrippsiella*, it proved helpful to store droplets of the cultures in a wet chamber for a few hours. Under these conditions many of the cells shed their thecae, to which the hypochlorite treatment was then applied. After testing other methods, the following technique was adopted for the examination of *Fragilidium*. Actively swimming individuals were killed by transferring them into 5% formaldehyde with a micropipette. Individual specimens were next isolated, and by applying gentle pressure to the cover-slip, the protoplasm was

[1] Contribution from the Scripps Institution of Oceanography, New Series.

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Scrippsiella sweeneyi n. gen., n. sp.

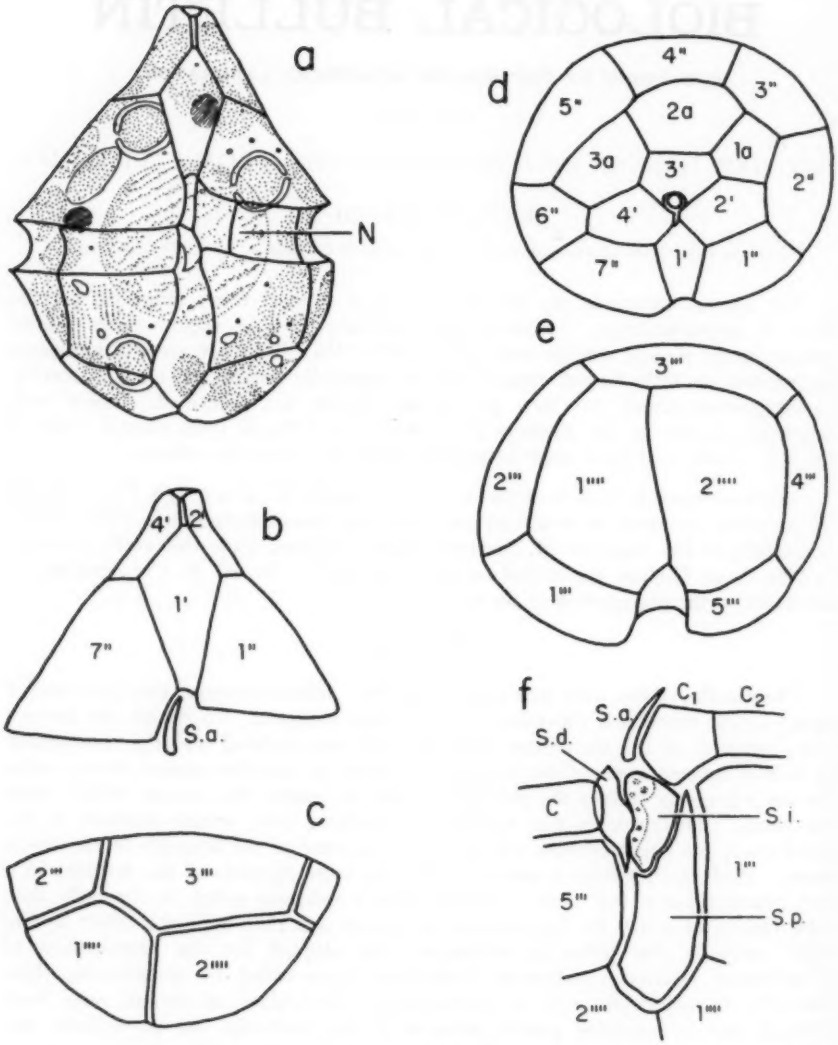

FIGURE 1. *Scrippsiella sweeneyi.* a) A typical individual, ventral view. b) Ventral view of the epitheca. c) Dorsal view of the hypotheca. d) Apical view of the epitheca. e) Antapical view of the hypotheca. f) Sulcal region (S.a.: Anterior sulcal.—S.i.: left sulcal.—S.d.: right sulcal.—S.p.: posterior sulcal). All figures about × 1500.

forced out of the theca through the cingular region. The hypochlorite treatment was then applied to the empty *Fragilidium* theca, especially in studies of the sulcus and cingulum.

Diagnosis. Small-sized, conical epitheca, rounded hypotheca, without horns. Cingulum wide, cavazone, descendent, with displacement equal to two-thirds of its width, without lists. The cingulum has six plates, five equal, preceded at the left by a transitional one. Sulcus deep, of medium width, slightly curved to the right.

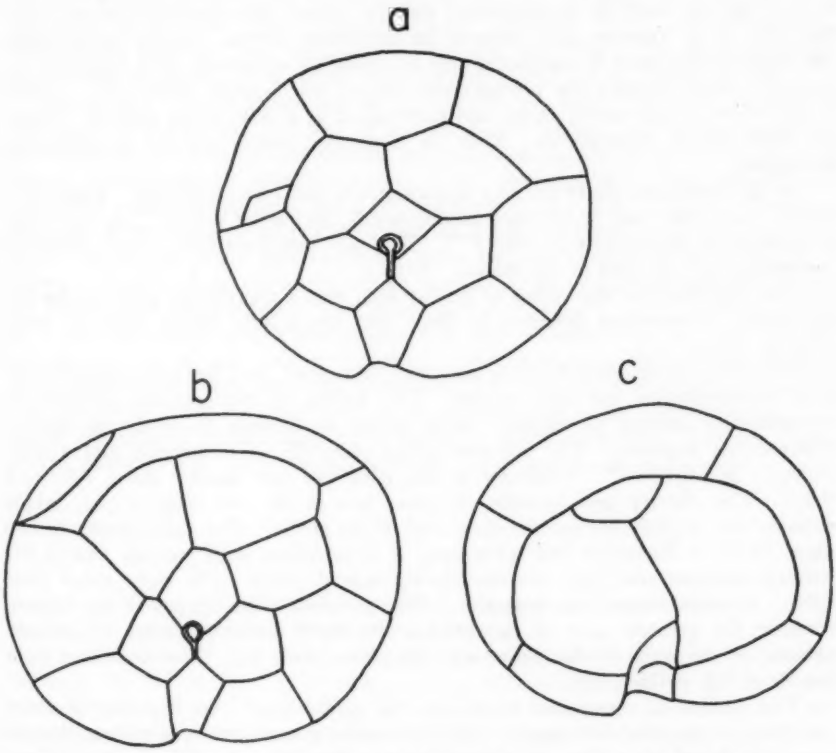

FIGURE 2. *Scrippsiella sweeneyi*, atypical plate patterns. a and b) Two epithecae, apical view. c) Antapical view of a hypotheca. All figures about × 1500.

The sulcus has four plates, with the posterior plate largest. The pattern of the major body plates is the same as that of an *Orthoperidinium* with three intercalaries. Cell length, 24–32.5 μ; transdiameter, 19–24 μ, chromoplasts numerous, elliptical, generally brown-yellow. La Jolla, California.

Description. This organism resembles *Peridinium trochoideum* in its general shape and size, and to some degree in its plate formula: 4′, 3a, 7″, 6c, 5‴, 2⁗, and 4s. Its epitheca is high and conical, most individuals deviating from a

rectilinear outline by a concavity near the apex, as shown in Figure 1a. The hypotheca is almost hemispherical, and slightly shorter in length than the epitheca. In the region of the girdle, there is a slight dorsiventral compression. In apical view, the cells normally appear almost circular. The sulcus indents slightly into the epitheca, is very deep, and of medium width. It does not reach the antapex when in true frontal view.

The plate pattern of the major body plates is the same as that of an *Orthoperidinium* with three intercalaries. In the epitheca, the first apical plate (1′) is very narrow, with an asymmetrical rhombic shape and upwardly curved base. Attached to its anterior end, there is an extremely narrow ventral apical plate. The apex of the theca is horizontal, and is closed by a circular plate (apical pore platelet) which indents the pentagonally shaped third apical plate (3′). Plates 2′ and 4′ are comparatively large, and generally 2′ is a little wider than 4′. There are three dorsal intercalaries. Plate 2a is usually pentagonal but is sometimes hexagonal.

In the hypotheca, there are five post-cingulars and two antapicals. Plates 1″ ′ and 5″ ′ are wide, and 3″ ′ is very asymmetrical; its border with 2″ ″ is very long in comparison with its border with 1″ ″. The two antapicals have a very restricted connection with the end of the sulcus.

The cingulum has five plates of similar size, plus a transitional plate at the left end which is somewhat different in shape and also a little higher than the other cingular plates.

The sulcus of dinoflagellates is not easily examined, and has been neglected by most protistologists for that reason. The sulcus of *Scrippsiella sweeneyi* is exceptionally difficult to analyze, being about as difficult to study as that of *Heterocapsa triquetra*. The anterior sulcal plate (S.a.) is narrow and a little curved. It borders 7″. Posterior to this plate are two smaller plates (S.i. and S.d.). The shorter and broader of these two is the left plate (S.i.), which extends very slightly beyond the distal end of the girdle. The right border of this plate (S.i.) is thickened and refringent; it is provided with poroids and at the extreme anterior end there are two closely spaced pores. The right sulcal plate (S.d.) narrows toward the posterior. The posterior plate (S.p.) is the largest, forming the greatest part of the sulcus. Its right anterior border is strongly oblique to the axis of the plate and articulates with S.i. The posterior right border of S.p. is thickened.

The nucleus is round and located at the girdle level. Its diameter is about one-third of the total cell length. The chromatin strands are less evident than in most dinoflagellates. The chromoplasts are elliptical and numerous, sometimes yellow-green but normally brown-yellow. Food is apparently stored as small granules and also around the chromoplasts in bodies that resemble pyrenoids. There is no pusule nor stigma.

The first external evidence of cell division is the formation of two discrete longitudinal flagella with separate points of attachment. During division, the cell escapes from the theca but retains a tough cellular membrane. The two daughter cells remain attached to each other in an oblique plane. The posterior cell is usually the smallest.

Locomotion is normally rapid, with a strongly rotatory motion. There is usually

one complete rotation of the organism during an advancement of one or two cell lengths. Sometimes, when *S. sweeneyi* cells reach the border of a drop, they suddenly cast off their flagella. Generally they lose the transverse ribbon-like flagellum first, which continues to beat in the detached state for a few seconds and then vacuolizes. The longitudinal flagellum is about three times as long as the cell; it does not beat or vacuolize after detachment.

Occurrence. This organism was originally isolated on March 15, 1956, from water collected off the S.I.O. pier at La Jolla, California, and has since been observed frequently in locally collected water samples. It seems to be a year-round inhabitant of the San Diego region, thriving especially in the summer months. This species has also been observed in plankton net samples, its relative scarcity in these being caused by its small size and poor retention on plankton silk.

Variations. The cingular and sulcal formula has been constant in laboratory and field specimens: 6C and 4S. The cells varied in size, and in the laboratory clonal culture used in the description, the cells also varied in shape and in plate pattern. Deformed or aberrant forms of *S. sweeneyi* were numerous in old laboratory cultures, but these were not observed in plankton samples. The plate formulae of the thecae from plankton samples were established in only a few cases, so we do not yet know how much the plate formula of this organism varies in nature. On the whole, the plate pattern has shown an amazing range of variation.

The normal plate formula is as stated above: 4′, 3a, 7″, 6c, 5‴, 2‴″, and 4s. It is generally assumed that the hypotheca is more conservative than the epitheca, and this is true of the present organism. Deviations from the normal epithecal plate configuration were observed in about 10 per cent of the specimens examined. The range of variation encountered in the atypical specimens of *S. sweeneyi* was rather exceptional for dinoflagellates, although similar variations occur in *Pyrophacus horologicum*. Plate formulae in these atypical specimens were:

(1) 4′, 2a, 6″;
(2) 4′, 3a, 5″;
(3) 4′, 3a, 4″;

A single specimen with 3′, 3a and 5″ showed an exceptional overgrowth of 1″, which reached the apical pore thus transforming 2′ into 1*a*.

No alteration of hypothecal formula has been noticed in actively growing cultures. In old cultures, I have observed hypothecal formulae of: 5‴, 2‴″ and one intercalary; 4‴, 2‴″; and 3‴, 2‴″ and one intercalary. However, the plate variation of the hypotheca has not exceeded two per cent in all examined specimens.

Discussion. The general characteristics of this organism place it in the *Peridiniaceae*. If it were classified solely on the basis of its major body plates, it would be included in the genus *Peridinium*. The cingular formula and sulcus plates are characteristically different, however, from those of *Peridinium*. The cingular and sulcal plates are conservative and important structural features connected with the most dynamic parts of the cell. Undoubtedly this organism belongs to a new genus. The species is also new. The only other known species which bear general resemblances to the present organism are *Peridinium subsalsum* Ost., and especially *P. trochoideum* (Stein) Lemm. Laboratory cultures of these species were provided by Dr. Sweeney for comparative studies. Their assignment

to the genus *Peridinium* was clearly correct, but they bore only superficial resemblances to *Scrippsiella sweeneyi*. The genus is named after the institution at which it was discovered, and the species is dedicated to Dr. Beatrice M. Sweeney who made the original isolation and whose cultures made this study possible.

Fragilidium heterolobum n. gen. n. sp.

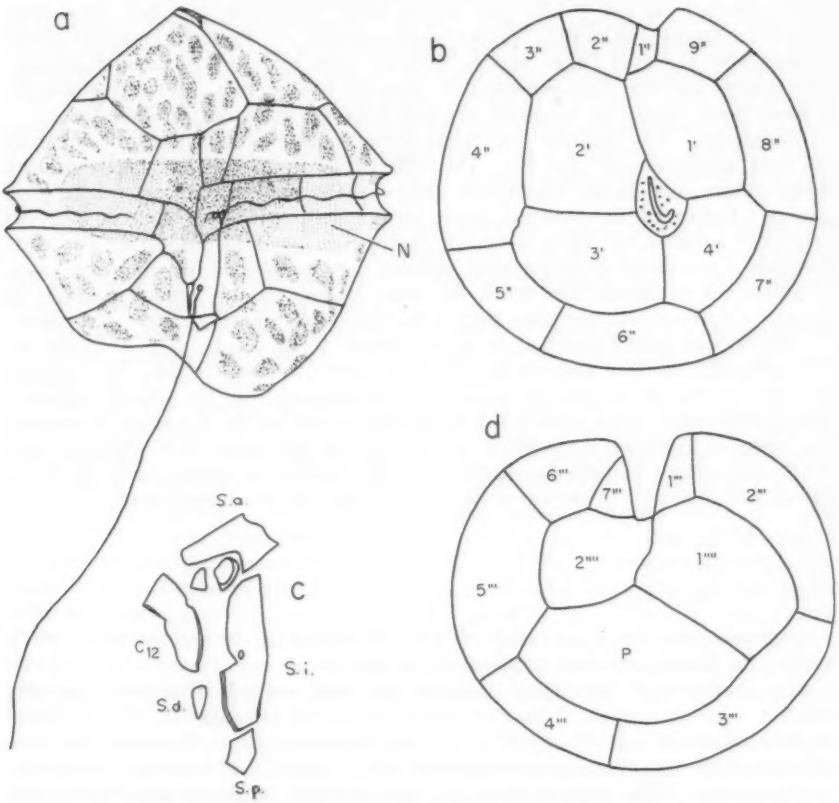

FIGURE 3. *Fragilidium heterolobum*. a) A typical individual, ventral view. b) Apical view of the epitheca. c) Sulcal plates. d) Antapical view of the hypotheca. All figures about × 1000.

Diagnosis. Medium-sized, roughly roundish pentagonal in ventral view. Epitheca dome-shaped; hypotheca asymmetrically bipedal, the left lobe being the largest. Cingulum deeply impressed, subcentral, descending, displaced distally about one girdle width, without lists. The cingulum has eleven sub-equal rectangular plates plus a transitional plate at the right end. Sulcus narrow, only slightly excavated, with six plates. Theca easily exuviated. Cell length 53–56 μ,

transdiameter 48–54 μ. Chromoplasts numerous, elliptical, brown. Genus characterized by the high number of precingular, postcingular and cingular plates. La Jolla, California.

Description

Plate pattern. The epithecal formula is 4′, 9″ and a "pore platelet." The plate 1′ is in general large and it has the most irregular form. It has connections with seven plates: 1″, 2″, the pore platelet, 4′, 8″ and 9″; its border for 2″ is the smallest. Plate 1′ is asymmetrically located, most of it being on the right side of the epitheca; its width decreases gradually to the left. The other three apicals are more regular. Apical 2′ has six edges (for 1′, 3′, the pore platelet, 2″, 3″, 4″). The plate 3′ touches 2′, 4′, 4″, 5″, 6″ and the pore platelet. The apical 4′ touches 3′, 1′, 6″, 7″, 8″ and the pore platelet.

The so-called pore platelet is relatively large, oval sigmoid, and placed obliquely to the median plane, *i.e.*, the plane which passes through the sulcus, the joint of 1″ and 9″, and the apex. This plate is variable but generally it has a convex left side subdivided into two edges for 2′ and 3′, a concave side which touches 1′, a major pole for 4′, and a minor one for the suture between 1′ and 2′. The most characteristic feature of this plate is a long and narrow reinforcement at the middle of the plate, sigmoid, with a dorsal hook to the right; it is variable sometimes double. Along it there are sometimes a few very small pores.

The most characteristic precingulars are 1″ and 9″. The first, trapezoidal in shape, is the smallest. The precingular 9″, pentagonal, has two edges at the left: the superior one for 1′ and the posterior, reinforced, forms a part of the right border of the sulcus. The precingulars 3″, 5″, and 7″ are more or less quadrangular.

The hypothecal formula is 7‴, 2‴ and 1p. The narrowest postcingular plates are 1‴ and 7‴. The latter is the smaller and is somewhat displaced posteriorly. The antapicals are very asymmetrical, the left one being much longer. The suture between 1‴ and 2‴ is irregular. Antapical 2‴ contacts the sulcus more than 1‴, which just barely touches it. The intercalary (p) is a large irregular plate, bordered by the antapicals, 3‴, 4‴ and 5‴.

All plates of the epi- and hypotheca are smooth. Some spots of different optical densities could be seen in a few specimens, especially in plate 1″, with oil immersion and phase contrast. There are sometimes pores located on the cingular border of this plate.

The cingulum is formed by eleven subequal retangular plates, plus another different plate at its right end. This C_{12} is curved, irregular, extending somewhat into the sulcus, with a narrow left-posterior or sulcal end. For that reason this plate could be named "transitional." The cingular plates lack sculptures.

The sulcus is narrow, has six plates, and is only slightly excavated. The anterior sulcal plate has a very characteristic "boomerang" shape, with a posterior concavity and a longer and narrower right arm. In its sinus there are two very tiny platelets; the right one is the smaller. Behind the anterior plate and in contact with 1‴ there is a long plate, with a little sinus at the middle of its right border, where C_{12} ends. In connection with the latter, there is another small plate. Finally, there is a posterior sulcal plate.

Protoplasm. The protoplasm is surrounded by a strong membrane and contains more than one hundred elongate-elliptical chromatophores which are dark

yellowish-brown. Food is stored as numerous granules of variable shape, which are generally small and located most abundantly in the peripheral layer.

The nucleus is large and compact, and is surrounded by a strong membrane. It is elongated in the equatorial plane, is somewhat curved, and has very dense thin threads of granular chromatin more or less perpendicular to the major axis of the nucleus. At the concavity, I sometimes observed large masses that were not distinctly granular.

The longitudinal flagellum extends beyond the antapex about two and a half cell lengths; it has a fast vibratory movement of short amplitude. The transverse flagellum, very slightly flattened, is long and completely encircles the girdle. The organism swims with a predominantly rotating motion.

Dimensions (in fixed and slightly distorted cells). Length 53–56 μ; transdiameter 48–54 μ. In an individual with a length of 55.5 μ, the epitheca was 27.5 μ and the hypotheca was 24 μ in length.

Variations. I have observed some variation in form (cell length more or less short in comparison with the transdiameter) and also in the plate pattern. Sometimes 4″ appears divided into two plates; thus the postcingular series sometimes has eight instead of seven plates. Occasionally there are eight instead of nine precingulars, and in one individual, a very narrow 1′ was observed fused with the pore platelet.

The normal formula is: 4′, 9″, a pore platelet, 12c, 7‴, 2⁗, 1p and 6s.

Discussion

The only difficulty encountered in the tabulation of this organism was the rapidity with which it exuviated its plates. Most of the individuals were found in a quiet state, short ellipsoidal in form, and without theca. The actively swimming cells were of course difficult to measure and draw. Any attempt to stop them for a moment led to cell deformation and ecdysis. This is accomplished in a very peculiar way: all the plates separate from each other, but in general, they remain surrounding the cell at a short distance, forming a regular assemblage. The plates are very delicate.

The plate pattern of this species is fundamentally different from all of those previously known (Balech, 1956; Biecheler, 1952; Dangeard, 1927; Graham, 1942; Kofoid, 1907–33; Lindemann, 1928; Schiller, 1933, 1937). The differences are in both the epi- and hypotheca. Since very little is known regarding the cingulum and sulcus of most dinoflagellates, we cannot discuss the differences concerning these regions. Nevertheless, it should be pointed out that the structure of the sulcus of this species is different from that of all sulci already studied, and no other genus is known with such a high number of girdle plates.

Other genera without epithecal intercalaries and with four apical plates are *Diplopsalis, Dolichodinium, Goniodinium, Glenodinium, Cladopyxis,* and *Ceratium.* The two latter are very different in form, bearing strong horns or arms, and with many differences in plate pattern. *Diplopsalis* as defined by Lindemann (1928) is actually an assemblage of several genera. But even on these terms, *Diplopsalis* never has more than seven precingulars, five postcingulars and it lacks the posterior intercalary. *Glenodinium,* as defined by Schiller (1933–37), is another very heterogeneous assemblage with a very large variation of plate patterns. None of its

species has so many pre- and postcingular plates, and they also lack posterior intercalaries. *Dolichodinium* seems to have only six girdle plates and has six precingulars and six postcingulars instead of nine and seven. *Goniodinium* is perhaps the genus most closely related to *Fragilidium*, but it has only six precingulars and six postcingulars; instead of one posterior intercalary it has three intercalaries in the hypotheca. Until the discovery of *Fragilidium*, *Goniodinium* was the genus with the highest known number (nine) of cingular plates (this number, however, was not stated with certainty).

The high number of precingular, postcingular and cingular plates is sufficient to characterize this new genus. The only other genera with seven postcingulars are *Glenodiniopsis* and *Heterodinium*. *Pyrophacus* is the only genus with a higher number of these plates.

Fragilidium heterolobum was isolated from plankton at La Jolla (San Diego, California) on March 20, 1957.

Summary

1. Two new genera and species of dinoflagellates are described. Both were originally isolated from plankton samples collected at La Jolla (San Diego, California).

2. *Scrippsiella sweeneyi* is a small species with the general tabulation of an *Orthoperidinium*, but it differs in having six cingular plates. The structure of the sulcus is also different. A great deal of variation in plate pattern was exhibited by this organism.

3. *Fragilidium heterolobum* is a medium-sized species having a tabulation that is quite different from all previously described dinoflagellates. It has a very high number of cingular plates (twelve). The generic name refers to the characteristic frequency and suddenness with which it sheds its plates.

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PHOTORECEPTION IN THE OPOSSUM SHRIMP, MYSIS RELICTA LOVÉN [1]

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Little information is available on the physiology of photoreception in the Mysidacea other than a few studies of phototaxis. Practically nothing is known of their spectral sensitivity, dark-adaptation, or lower limits of vision. The two observations that have been made on the response of the opossum shrimp, *Mysis relicta* Lovén, have shown that they swim downward when subjected to light (Dakin and Latarche, 1913) and are especially sensitive to a combination of high temperature and bright light (Larkin, 1948). The present study of the spectral sensitivity, dark-adaptation, and phototaxis of *M. relicta* was undertaken to add to our information on the physiology of photoreception.

Materials and Methods

Mysis relicta is ideal for a study of this nature, since its large size (average length, 15.0 mm.) facilitates observation, and a laboratory population can be easily maintained. The animals used in this study were collected in Lakes Huron and Michigan.

All experiments were conducted in a cold-room under controlled light conditions and at a constant temperature of 10° C. The dark-adaptation and special sensitivity studies were carried out in an all-glass aquarium 12.0 by 8.0 by 3.0 inches, filled to 1 inch from the top. A 24-inch glass tube, with a 1-inch diameter, was used in the experiments on phototaxis.

Under conditions of continuous darkness or light, the mysids normally rested on the bottom of the tank, periodically making short excursions off the bottom. If the experimental light was turned on when the mysids were swimming upwards, they hesitated momentarily, turned, and swam rapidly to the bottom of the aquarium. This momentary hesitation was found to be a reliable indicator for the photic response.

The difficulty of observation in a dark room was met by using an infra-red viewer and infra-red light source or a large Fresnel lens to focus low-intensity red light (approximately 0.001 foot-candle) on the observer's eye. The red light was produced by a neon glow lamp and a number 2404 Corning glass filter, transmitting wave-lengths of 620 mμ and greater. It was permissible to use this light for viewing, since preliminary studies had established that *M. relicta* was almost completely insensitive to the red region of the visible spectrum in the 620-

[1] Based on a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree, University of Michigan, 1958.

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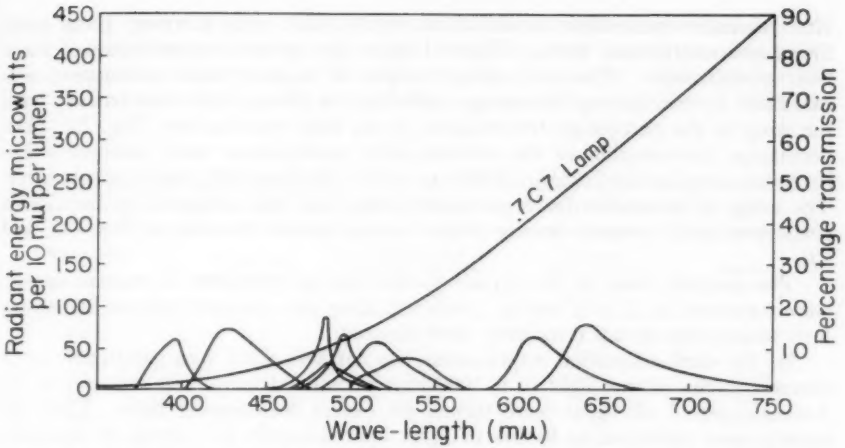

FIGURE 1. Spectral distribution of a tungsten filament lamp (color temperature 2300° K) and the spectral transmission of monochromatic filter combinations.

to 700-mμ wave-band. Mysid behavior was the same when viewed by either of the above methods.

A General Electric 7C7 tungsten filament lamp suspended 3 inches above the water surface provided the light source for the spectral sensitivity studies. This lamp has an average color temperature of approximately 2300° K and its lumen output averages 45. Although the exact distribution curve of spectral energy was not available for the lamp, a reasonably accurate curve was constructed by extrapolation from data supplied by the Nela Park Laboratory of General Electric (Fig. 1).

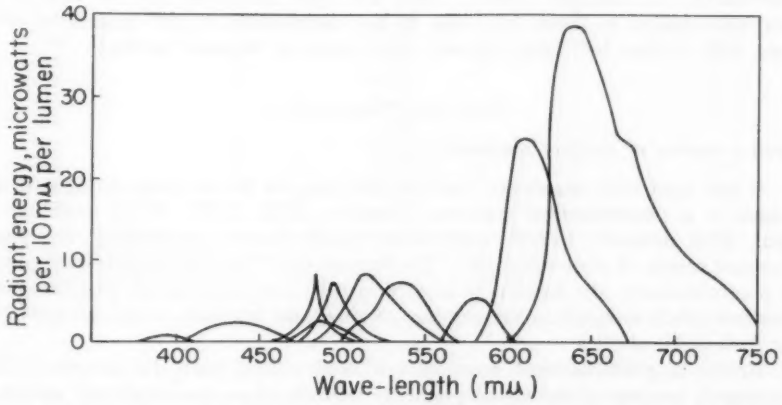

FIGURE 2. Radiant energy output from a tungsten filament lamp (color temperature 2300° K) through various monochromatic filter combinations.

Nine monochromatic-filter combinations were made with Corning glass color filters and interference filters; Figure 1 gives the spectral transmission of these filter combinations. The total energy output of a given filter combination was calculated by multiplying the energy available (in 10-mμ-wide wave-bands) from the lamp by the percentage transmission of the filter combination (Fig. 2). The percentage transmission of the various filter combinations was obtained either from data supplied by Corning (1948) or with a Beckman DU Spectrophotometer. The range of intensities for a particular wave-band was obtained by the use of evaporated-metal, neutral-density filters, having optical densities of 0.6, 0.9, and 1.0.

The reaction times of the mysids to the various intensities of radiant energy were measured by a stop watch. Sufficient time was allowed between successive tests to keep the mysids completely dark-adapted.

In the dark-adaptation experiments, the mysids were first subjected for 3 minutes to the intense light of a 1000-watt photoflood lamp. At the end of the 3-minute period all lights were turned off except the viewing light. Then the mysids were subjected to flashes of light, approximately 0.1 second in duration, of a given intensity. These flashes were spaced at 1- to 3-minute intervals. The experimental light was a 6-watt, 9-foot-candle, 115-volt, tungsten-filament lamp suspended 3 inches above the water surface. The intensity of the light was altered by interposing various numbers of evaporated-metal, neutral-density filters, each having an optical density of 1.0. The time in the dark, prior to first stimulation, was measured by stop watch.

In the experiment on the phototactic response of *M. relicta,* six individuals were placed in a 24-inch glass tube, with a 1-inch diameter, lying horizontally to eliminate any gravitational effects. The experimental light, a 7C7 lamp, was suspended 1 foot above the midpoint of the tube. After the mysids had been subjected for measured intervals to total darkness or light, one-half of the tube was shaded and the number of mysids in the unshaded half of the tube was recorded at 30-second intervals for a 5-minute period. First the right and then the left half of the tube was shaded to detect any bias in the distribution of the mysids. Control runs, with neither half being shaded, were made at frequent intervals.

Spectral Sensitivity

Earlier studies of spectral sensitivity

It has been well established that the first step in the response to light in any animal is a photochemical reaction. Hecht's (1919, 1920, 1921) work on the clam, *Mya arenaria* Verrill, contributed much toward establishing the photochemical nature of photoreception. He demonstrated that the fundamental concept of photochemistry, the Bunsen-Roscoe reciprocity law which holds that the photochemical effect is equal to the product of time and intensity, could be applied to the data of his studies.

Before a photochemical reaction can occur, light must be absorbed. The absorption spectra of the visual pigments will therefore determine the effects on the photoreceptor and in turn the response of the organism. The behavioral response of an organism to different regions of the radiant-energy spectrum has

given rise to the concept of "action spectra." For example, if a visual pigment has its absorption peak at a wave-length of 500 mμ, the animal possessing this visual pigment would be most sensitive to this wave-length. This concept has led to a number of action-spectrum studies on invertebrates. Mast (1917) re-

TABLE I

Reaction time (seconds) of Mysis relicta to various wave-lengths of light at several intensities (Unless noted otherwise the neutral-density filters transmitted 10 per cent of the light)

Wave-length of peak transmission (mμ)	Number of neutral-density filters	Energy output of 7C7 lamp through filters (microwatts/10 mμ/ lumens)	Average reaction times for 10 trials (seconds)	Standard deviation
395	0	2.20	0.77	0.18
395	*1	0.26	0.88	0.12
395	2	0.02	1.16	0.39
430	0	11.19	0.72	0.06
430	**1	2.80	0.84	0.06
430	*1	1.34	0.86	0.31
430	***2	0.34	0.92	0.16
430	****3	0.03	1.15	0.39
485	0	7.63	0.87	0.14
485	1	0.76	1.04	0.12
485	2	0.08	1.18	0.10
488	0	13.63	0.86	0.19
488	2	0.14	1.13	0.20
496	0	14.93	0.77	0.24
496	1	1.49	0.82	0.11
496	2	0.15	0.98	0.14
515	0	32.98	0.64	0.13
515	1	3.30	0.71	0.20
515	2	0.33	0.83	0.21
515	3	0.03	1.01	0.19
515	4	0.003	1.42	0.36
540	0	29.85	0.84	0.15
540	1	2.99	0.92	0.06
540	2	0.30	0.95	0.22
540	3	0.03	1.33	0.25
610	0	108.94	1.10	0.17
610	1	10.89	1.10	0.09
610	2	1.09	1.33	0.36
640	0	256.11	1.13	0.31
640	1	25.61	1.22	0.43
640	2	2.56	1.32	0.45
640	3	0.26	(No reaction)	...

* Neutral-density filter transmitting 12 per cent of the light.
** Neutral-density filter transmitting 25 per cent of the light.
*** Filters 1 and 2 combined.
**** Neutral-density filter transmitting 10 per cent of the light combined with filters 1 and 2.

ported peak sensitivity to light at the following wave-lengths: 483 mμ in *Euglena, Arenicola, Trachelomonas,* and *Lumbricus;* 524 mμ in *Pandorina* and *Eudorina;* 503 mμ in *Chlamydomonas,* and blowfly larvae. Hecht (1921) established the basis for future studies through his experiments on *Mya arenaria.* This clam

had a maximum sensitivity at 490 mμ. Most insects apparently have two peaks of maximum sensitivity, 365 mμ and 492 mμ (Weiss, 1943).

Results obtained through the methods of these investigators are similar to those secured by *in vitro* studies of the spectral absorption of squid rhodopsin (Bliss, 1948). An electrical method has been employed to determine the spectral sensitivity of the eyes of *Limulus* (Graham and Hartline, 1935), a grasshopper, *Melanoplus* (Jahn, 1946), and the diving-beetle, *Dytiscus* (Jahn and Wulff, 1948). These studies demonstrated, as did the behavior method of Weiss, that the arthropod eye has a peak sensitivity in the green region of the spectrum.

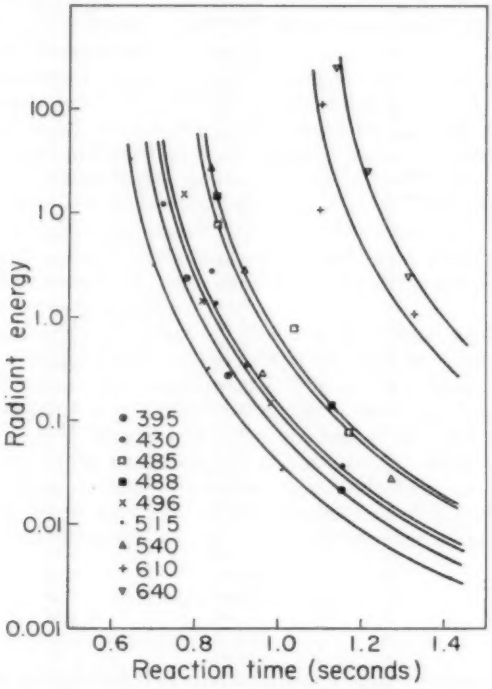

FIGURE 3. Reaction rate of *Mysis relicta* to certain wave-lengths (mμ) of light at various intensities. (Each point represents the average reaction time for 10 trials.)

The only source of information on the sensitivity of mysids to various regions of the spectrum is Hess' (1910) study of the phototactic response of a marine mysid to certain regions of the visible spectrum. Sixty-four mysids were placed in the dark and then subjected to a continuous spectrum of light. The mysids swam toward the light and aggregated in certain regions of the spectrum: 40 in the yellow-green, 19 in the blue and violet, and 5 in the red. When the position of the spectrum was altered the mysids followed the yellow-green band.

Spectral sensitivity in M. relicta

The dark-adapted mysids reacted to the different regions of the spectrum with the typical photic response but the time of reaction varied with wave-length. The mysids reacted most quickly at wave-lengths in the vicinity of 515 mμ and 395 mμ (Table I). The reaction times at wave-lengths of 610 mμ and 640 mμ were much slower (Fig. 3) despite the fact that considerably more energy was available in the red region of the spectrum than in the blue region (Fig. 2). If the mysid eye were equally sensitive to all regions of the spectrum, the faster reaction time

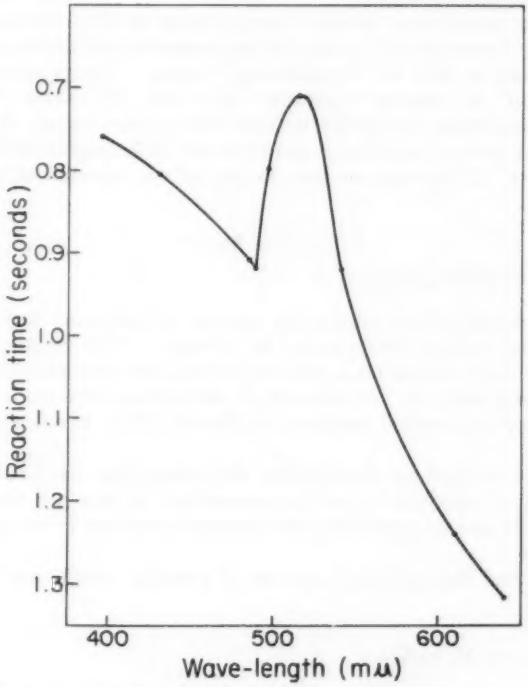

FIGURE 4. Action spectrum of the dark-adapted *Mysis relicta* eye.

would have been elicited by the higher energy of longer wave-lengths. The speed of response decreased progressively as the wave-length increased from 395 mμ to 488 mμ (Fig. 3). Then the trend was reversed and the mysids responded progressively faster as the wave-length increased. The reaction time at a wave-length of 496 mμ was similar to that recorded at 430 mμ and the quickest response was elicited by light with peak energy at 515 mμ. The reaction to light with wave-lengths longer than 515 mμ became progressively slower as the wave-length increased.

The reaction times of the mysids to a wide range of intensities for a particular

wave-length were determined through the use of evaporated-metal, neutral-density filters. Similar reaction times could be obtained for different wave-lengths by altering the intensity. Approximately 1000 times more radiant energy was required in the red region than in the blue-green region of the spectrum before equal reaction times could be obtained. The curves resulting from a plot of the reaction time against the radiant energy available from the 7C7 lamp and the filter combinations show that the reaction time varies inversely as the logarithm of the light intensity (Fig. 3). It is apparent that the rate of response to different wave-lengths was caused by differences in the ability of the eye to absorb energy at various wave-lengths.

The reaction times for a definite energy value of 3 as determined from the curves in Figure 3 were plotted against the transmission peaks of the monochromatic filter combinations to give an "equal-energy" curve. The resulting curve is the "action spectrum" or spectral sensitivity curve for *M. relicta* (Fig. 4). The mysids have a maximum sensitivity to light with a wave-length of approximately 515 mμ; another peak of sensitivity occurs in the violet region of the spectrum at or below 395 mμ. This suggests that the mysid eye contains at least two visual pigments.

Dark-Adaptation

Earlier studies of dark-adaptation

All of the photoreceptors studied by various investigators have shown an increased sensitivity to light after a period in darkness. This reaction has given rise to the concept of dark-adaptation, a process that has been studied by several different methods, including behavior. Studies on *M. relicta* have been made by a procedure similar to a behavioral method employed by Hecht (1919) to study dark-adaptation in *Mya*.

The behavior method for determining dark-adaptation has produced data that are similar to those obtained by *in vitro* resynthesis of bleached rhodopsin (Chase and Smith, 1939) and by measuring the electrical response of the eyes of *Limulus* (Hartline, 1930).

I could not find any published account of previous studies on dark-adaptation in Mysidacea.

Dark-adaptation in M. relicta

The 3-minute exposure of the mysids to the photoflood lamp evidently caused a bleaching of the visual pigment. The mysids did not respond to any light for at least 30 seconds after the lamp was turned off. Response to the experimental light after a period in darkness would indicate, therefore, that sufficient visual pigment had been resynthesized for photoreception. The light intensity necessary to elicit a response after a period in the dark was considered the "threshold intensity."

The light intensity required to produce a reaction decreased with the increase of time spent in the dark (Fig. 5). Several repetitions of this experiment gave closely similar results. The increase in sensitivity was relatively fast during the first 57 seconds, and then somewhat slower. The upper part of the curve probably lies too far to the right, since the mysids did not start to swim up until at least

30 seconds after the photoflood light was turned off; consequently, it was impossible to determine their sensitivity during this period. Possibly the steeper slope in the upper part of the curve can be attributed to a state of shock from exposure to the intense light. The initial recovery of sensitivity of the mysid eye, therefore, may progress at a rate other than indicated by the upper part of the curve in Figure 5. Light intensity values of 9×10^{-2} foot-candle and below

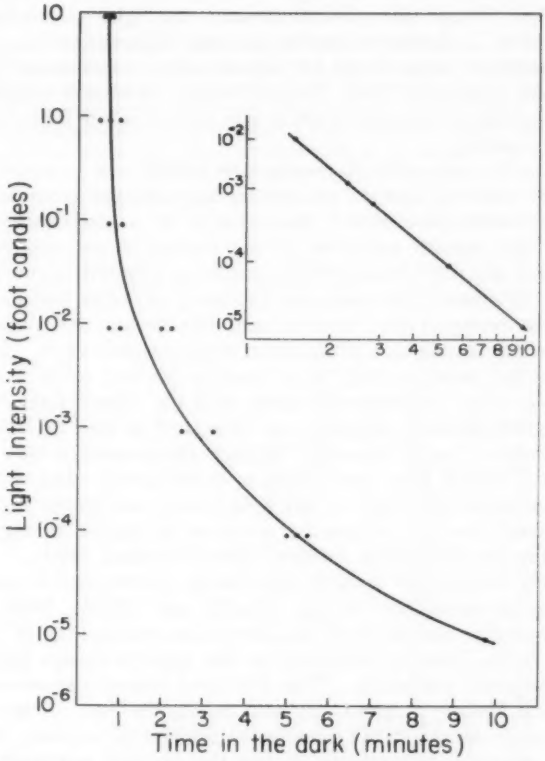

FIGURE 5. Dark-adaptation of *Mysis relicta*. Threshold intensity is plotted as ordinate against time in the dark as abscissa. Inset is the plot of log of threshold intensity against log of time in the dark.

and time in the dark gave a straight line when plotted on a logarithmic scale. These curves closely resemble that for a bimolecular reaction.

Although a precise determination of the lowest limits of visual sensitivity could not be made, a definite response to a light intensity of 10^{-6} foot-candle was established. In all probability the mysids can detect even a much weaker light.

The question arises as to whether the stimulating flashes themselves have any effect upon the dark-adaptation of the eye. Hartline (1930) believed that if they are widely spaced in time, 3 to 5 minutes, they have little effect.

Phototaxis

Earlier studies on phototaxis

Photoreception is important for the normal orientation of mysids. Delage (1887) established that the statocysts of mysids have a balancing function, but it was subsequently demonstrated that if the statocysts are removed, the eyes make possible normal orientation in the light (von Buddenbrock, 1914). The mysids always kept their dorsal side oriented toward the light—a reaction termed the "dorsal light reflex." Additional studies on three different species of mysids have shown that orientation is governed by responses to a combination of gravitational and light stimuli (Fraenkel, 1931; Foxon, 1940). Fraenkel suggested that light stimulates the mysids to assume a position whereby a definite region of the statocyst is stimulated by gravity.

The phototactic responses of mysids are varied and complex. Keeble and Gamble (1904) reported that the phototactic sign changed from positive to negative when the mysids were moved from a white to a black background. Bauer (1908) found that mysids remained at the bottom of an aquarium when light came from above and were positively phototactic to a lateral light. Menke (1911) concluded that *Hemimysis lamornae* was positively geotactic and negatively phototactic. He also removed the statocyst and interpreted the mysids' subsequent failure to leave the bottom as a pronounced negative phototaxis. Recent research has shown that this behavior may be a "general position reflex" whereby mysids utilize the tactile sense for orientation along with the "dorsal light reflex" (Foxon, 1940). Some differences in response can be related to age. Adults of *Neomysis vulgaris* [*N. integer* Leach] are more strongly photonegative than are the young (Lucas, 1936). Mysids have been shown to be telotactic; when two light rays are crossed at right angles they react to one light source and ignore the other. Light-adapted individuals also have shown a reversal in the phototactic sign by swimming to and fro in the direction of the incident light (Fraenkel, 1931).

The observations of other workers that certain marine mysids possess a diurnal rhythm in their chromatophore system (Keeble and Gamble, 1904) suggested the possibility of a similar periodicity in the phototactic responses of *M. relicta*. Their investigations of the chemical conditions of the mysid's tissues indicated the possibility of a metabolic periodicity. The liver and muscle tissues were alkaline in the morning and became progressively acid during the day. Some of the experimental work which Menke (1911) conducted led him to postulate that *Hemimysis lamornae* was negatively phototactic during the day and positively phototactic at night. The experiment described in the next section was devised to detect such a periodicity in *M. relicta*. Although the results were not conclusive on that point, other important findings warrant inclusion of these data in this paper.

Phototaxis in M. relicta

Whenever the control runs were conducted the distribution did not differ significantly from random. The distribution did differ significantly (Chi Square test) when one-half of the tube was shaded after the mysids had been in the dark or light for a period of time. The mysids were photopositive unless they had been subjected to total darkness for 10 hours; then they became photonegative

(Table II). The photonegative condition persisted for only a short time as they became light-adapted within 6 minutes. On August 15 at 11:54 Eastern Standard Time (EST) the mysids were definitely photonegative after being in the dark for 13 hours; after exposure to the experimental light for 6 minutes they tended to be photopositive. On the same date at 21:06 EST the mysids still responded photopositively after approximately 4 hours in the dark. Similar results were

TABLE II

Phototactic response of M. relicta to a constant light intensity after periods in light and in total darkness. [Observations were made of the distribution of 6 animals at 30-second intervals for 5 minutes, except at 21:12 (5½ minutes), 11:55 (2½ minutes), 11:58 (2½ minutes). Numbers in parentheses indicate control run, i.e., entire tube unshaded. Asterisk indicates significant Chi Square value at 5 per cent level. Experiments were started at 20:54 EST on August 14, 1956, and completed at 12:06 EST on August 18, 1957]

Time (EST)	Time in dark		Time in light		Number in portion of tube		Chi Square
	Hours	Minutes	Hours	Minutes	Shaded	Unshaded	
20:54	—	—	2	54	18	42	9.6*
11:54	13	5	—	—	51	9	29.4*
12:03	—	—	—	6	25	35	1.66
13:05	—	—	1	8	9	51	29.4*
13:11	—	—	1	14	17	43	11.27*
13:37	—	21	—	—	27	33	0.6
16:38	—	—	—	—	(30)	(30)	0.0
16:51	—	—	2	56	19	41	8.06*
21:06	4	10	—	—	12	48	21.6*
21:12	—	—	—	6	28	38	1.51
21:17	—	—	—	—	(31)	(29)	0.066
10:19	12	57	—	—	36	24	2.4
10:25	—	—	—	6	43	17	11.26*
10:31	—	—	—	—	(28)	(32)	0.266
12:13	1	36	—	—	34	26	1.066
12:19	—	—	—	6	17	43	11.26*
12:24	—	—	—	—	(29)	(31)	0.066
22:43	10	14	—	—	41	19	8.06*
22:48	—	—	—	5	35	25	1.66
21:28	3	38	—	—	26	34	1.06
21:34	—	—	—	6	21	39	5.4*
21:39	—	—	—	—	(34)	(26)	1.06
11:55	14	10	—	—	20	10	3.32
11:58	—	—	—	3	14	15	0.132
12:06	—	—	—	—	(33)	(27)	0.6

secured on August 16 at 10:19 EST and August 18 at 11:55 EST, the mysids were photonegative after 13 and 14 hours in the dark. At 22:43 EST on August 16 they were photonegative after 10 hours and 14 minutes in the dark. The fact that the mysids could be photonegative either in the morning or at night indicates that the phototactic response does not have a persistent diurnal rhythm. Existence of the photonegative condition depends on the amount of time in the dark.

Experiments revealed also that if the light intensity was increased rapidly by

1 or 2 foot-candles, the mysids swam into the shaded area, although they previously had been definitely photopositive. They adapted to this increased intensity within 2 minutes. Johnson (1938) obtained similar results with the copepod, *Acartia clausii* Giesbrecht. These copepods were placed in a glass cylinder and subjected to a step-wide change in intensity. The more rapid the change in intensity, the greater was the response. The copepods were photopositive after a period in the dark, but they swam away from the light when the intensity was increased.

Discussion

The experiments indicate that *Mysis relicta* has at least two visual pigments, one with an absorption peak at 515 mμ and another with a peak at or below 395 mμ. The maximum sensitivity at 515 mμ probably is important to the mysid for orientation in the environment, since light with a wave-band of 490 mμ to 540 mμ penetrates to considerable depth in Lake Huron (Beeton, 1958). The importance of the sensitivity to violet light (395 mμ) is not obvious, since light with a wave-band of 300 mμ to 420 mμ does not penetrate into the deep-water habitat of the mysids. Ultraviolet light has been found to cause negative phototaxis in *Daphnia pulex* (Moore, 1912), and *D. magna* (Baylor and Smith, 1957). In view of the deleterious effect of ultraviolet light, it is not surprising to find that many arthropods are sensitive to short wave-lengths and photonegative to ultraviolet light.

The mysids are able to "dark-adapt" at a relatively fast rate. This increase in sensitivity is interpreted as a function of the amount of regenerated visual purple available at each moment in the dark. The curve describing the progress of this reaction resembles that for the equation for a bimolecular reaction. The literature, however, reveals considerable disagreement as to whether the dark-adaptation data are fitted best by the equation for a bimolecular or monomolecular reaction. The reaction is probably bimolecular, but appears monomolecular when one of the reactants is in excess. Some of the discrepancies in the results obtained by different workers may possibly be explained, in part, by the data of Haig (1941). Haig's data showed that the curve of recovery after adaptation to a low intensity is decidedly different from that of recovery after adaptation to high intensity of illumination. Hartline and McDonald (1947) presented a similar series of recovery curves for single visual elements of *Limulus* after exposure to light of different intensities.

The change in the phototactic response of *M. relicta* is related to the prior presence or absence of light. Time also is involved, since the mysids were photopositive unless they had been subjected to total darkness for 10 hours or more; then they responded photonegatively.

Changes in the phototactic sign may be related to the activity of the organism. The mysids were very active in the light and not as active in the dark; possibly the increased activity results in an acidic condition in the tissues. The acidity would decrease with reduced activity. *Daphnia*, copepods, *Gammarus* (Loeb, 1918), and *Hemimysis* (Franz, 1911) can be made positively phototactic by adding a weak acid to the water. The assumption of a metabolic change due to differences in activity does not account for the rapid change from a photonegative to a photopositive condition. The possibility remains that light may control a chemical cycle in the organism. A compound, responsible for the photonegative

condition, may be broken down upon exposure to light. It may require a long period of darkness for resynthesis of this compound.

Drs. David C. Chandler, Ralph Hile, and Stanford H. Smith reviewed the manuscript. Dr. E. R. Baylor lent items of equipment and made invaluable suggestions concerning the experiments.

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THE RESPIRATION OF UNFERTILIZED SEA URCHIN EGGS IN THE PRESENCE OF ANTISERA AGAINST FERTILIZIN [1]

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It has been shown (Tyler and Brookbank, 1956a, 1956b) that antisera against purified fertilizin, as well as antisera against homogenates of fertilized and unfertilized eggs deprived of the gelatinous coat material, increase the respiratory rate of demembranated fertilized eggs, and cause an inhibition of cleavage. The respiratory rate of blocked eggs increases to a maximum of 4 to 5 times the control rate in 20 to 40 minutes, and subsequently decreases to the rate of the controls during the next 40 minutes. Gradual cytolysis of the eggs occurs following 4–5 hours of exposure to undiluted antisera. The observed increase in the respiratory rate in the presence of immune serum is apparently without parallel in the previous literature. Haurowitz and Schwerin (1940) studied the respiration of pigeon erythrocytes in the presence of immune rabbit serum and active and inactive complement, yielding lysis and agglutination, respectively. No increase or decrease in respiratory rate was noted in either case. Sevag and Miller (1948), studying the respiration of *E. typhosa* (strain 0–901) in the presence of immune rabbit serum and active or inactive complement, found that intact, sensitized cells consumed oxygen at the same rate as the controls. However, in the presence of active guinea pig complement, the cells lysed, with an accompanying transitory increase (1.4-fold), which was followed by a decrease to one-fourth the control rate after 180 minutes. Harris (1948) measured the oxygen uptake of Salmonella cells in the presence of agglutinating rabbit serum, and found no increase or decrease in respiratory rate over a wide range of antiserum concentrations. Nowinski (1948) investigated the possibility of an effect on respiratory rate by reticulo-endothelial-immune-serum (REIS) acting on rat spleen slices, and by anti-chick brain serum acting on chick brain homogenates (1949). No effect on respiration was observed with REIS, and a slight inhibition of oxygen uptake of chick brain homogenates was noted in the presence of anti-chick brain serum. MacDonald (1949) obtained similar results with REIS using rat spleen slices in Thunberg experiments.

The purpose of the present experiments was to explore the effect of antisera against fertilizin, which is, chemically, a rather well defined substance (Tyler, 1949, 1956), on the respiratory rate of unfertilized sea urchin eggs. Unfertilized eggs, though normally respiring at a low rate, can be stimulated to respire at a much

[1] This investigation was supported in part by a research grant (RG 4659) from the National Institutes of Health of the United States Public Health Service. The author is also indebted to Professor Albert Tyler for a critical reading of the original manuscript.

[2] The author wishes to thank the Friday Harbor Laboratories of the University of Washington, Friday Harbor, Washington, for the use of space and equipment during the summer of 1957.

greater rate (4–5-fold increase) by parthenogenetic agents (Warburg, 1908; Keltch and Clowes, 1947), by nitrophenols (Clowes and Krahl, 1934, 1936) and other non-parthenogenetic substances, as well as by fertilization. In this connection, it is noteworthy that Perlman (1954, 1957), and Perlman and Perlman (1957) have reported that antisera against fertilizin, as well as antisera against extracts of unfertilized eggs, are capable of activating the unfertilized eggs of *Paracentrotus lividus*. The respiratory rate of eggs so treated therefore becomes of interest.

Materials and Methods

Preparation of antigens and antisera

1) *Lytechinus variegatus* (Cedar Key, Florida). Fertilizin antigens were prepared from neutralized supernatant egg-water of acid- (pH 3.5) treated unfertilized eggs. In the case of antigen number 4, the egg-water was dialyzed against distilled water and injected without further purification. Antigen number 10 was prepared by first precipitating the fertilizin of the egg-water (derived from a second spawn of eggs) with 5/4 volumes of cold 95% ethanol (Tyler, 1949). The fertilizin precipitate was then washed thoroughly with additional volumes of cold ethanol, and vacuum-dried. The dried precipitate was dissolved in distilled water, and used for injection. Both antigens had a final agglutination titer of approximately 1/1000 on homologous sperm.

A whole sperm antigen (number 11) was prepared from washed *Lytechinus variegatus* sperm (presumably free of seminal fluid) which were diluted to a 10% suspension by volume with sea water and frozen until used.

All above antigens were stored at − 20° C. in one-ml. aliquots. After thawing for use in injections and various tests, the material remaining in each individual aliquot was discarded. This procedure avoided repeated freezing and thawing of the antigens.

Antisera against the above antigens were prepared in rabbits according to an immunization schedule described elsewhere (Tyler and Brookbank, 1956a). Following a control bleeding, totals of 400 μg N (no. 4), 200 μg N (no. 10), and 6000 μg N (no. 11) were injected into the animals over a period of three weeks. The rabbits were bled by cardiac puncture 5 days following the final injection. The antisera were recovered from the retracted clots, and dialyzed thoroughly against sea water at 10° C., and stored at − 20° C.

In addition, an antiserum against extract of washed, demembranated, fertilized eggs of *Lytechinus pictus* was available, and was used in a number of experiments. This antiserum had been previously shown to increase the respiration rate of fertilized *L. pictus* eggs (Tyler and Brookbank, 1956b).[3]

2) *Strongylocentrotus purpuratus* (Friday Harbor, Washington). A single fertilizin antigen was prepared by precipitation of the fertilizin from the egg-water of acid- (pH 3.5) treated eggs by NaOH (in the ratio of 40 ml. 1 N NaOH per liter of egg water; Tyler, 1949). The resulting precipitate was dissolved in sea water, following neutralization of the alkali, and dialyzed against distilled water.

[3] Due to an oversight on the part of the authors, Tyler and Brookbank (1956b) contains an error on page 312, line 6. This line reads correctly if *L. pictus* is substituted for *S. purpuratus*.

TABLE I

The effect of antisera, normal sera, 0.1% sperm suspension, and hypertonic sea water on the respiration of unfertilized Lytechinus variegatus eggs

Experiment no.	Reagent employed	Aver. rate (µl./min./vessel)	Ratios of aver. rates (increased/control)
1	No. 4 normal serum	0.28	
	No. 4 normal serum	0.28	
	Anti-no. 4	0.67	2.4
	Anti-no. 4	0.67	
	Anti-*L. pictus* fertilized egg extract	0.73	2.6
	Anti-*L. pictus* fertilized egg extract	0.73	
	Buffered sea water	0.28	
	Buffered sea water	0.28	
	Buffered sea water	0.33	
2	No. 10 normal serum	0.25	
	Anti-no. 11 serum	0.31	
	Anti-no. 10 serum	1.18	4.1
	Anti-no. 10 serum	1.13	
	Buffered sea water	0.28	
3	No. 4 normal serum	0.33	
	No. 4 normal serum	0.28	
	Anti-no. 4	1.08	3.5
	Anti-no. 4	1.03	
	0.1% sperm suspension	1.07	3.5
	Buffered sea water	0.33	
	Buffered sea water	0.28	
	Buffered sea water	0.22	
4	No. 5 normal serum	0.30	
	No. 5 normal serum	0.22	
	Anti-*L. pictus* fertilized egg extract	0.92	3.4
	Anti-*L. pictus* fertilized egg extract	0.84	
	Buffered sea water	0.27	
5*	No. 11 normal serum	0.14	
	Anti-no. 10	0.95	6.7
	Anti-*S. purpuratus* fertilizin (three antisera)	0.37	2.6
		0.33	2.3
		0.33	2.3
	Buffered sea water	0.14	
6	0.1% sperm suspension	0.83	3.2
	0.1% sperm suspension	0.80	
	Hypertonic sea water	0.84	3.3
	Hypertonic sea water	0.84	
	Buffered sea water	0.25	
7	Anti-no. 10 undiluted	0.32	2.5
	Anti-no. 10 1:1 dilution	0.26	2.0
	Anti-no. 10 1:2 dilution	0.24	1.8
	Anti-no. 10 1:4 dilution	0.20	1.5
	No. 11 normal serum	0.16	
	Buffered sea water	0.10	
	Buffered sea water	0.12	

* In experiment 5, one ml. of 20% egg suspension was used with vessels of 7-ml. capacity, as opposed to 2.3 cc. of egg suspension with vessels of 15-ml. capacity in the other experiments. Five-tenths ml. of antiserum or normal serum was used throughout all experiments. The conditions in experiment 5 duplicate those which obtained during experiments with eggs of *S. purpuratus*.

After dialysis, the fertilizin was precipitated with 5/4 volumes of cold ethanol, washed, vacuum-dried, redissolved in distilled water, and used for injection into each of three rabbits. This antigen had a sperm agglutination titer of approximately 1/500.

Following a control bleeding, the rabbits were injected according to a previously described schedule (Tyler and Brookbank, 1956a) for a period of 2½ weeks, each receiving a total of 250 μg N. The animals were bled by cardiac puncture 4 days following the last injection.

Manometric methods

1) *Lytechinus variegatus.* The effect of the antisera on the respiration of unfertilized eggs, deprived of all soluble fertilizin by acid (pH 3.5) treatment, was followed, at 20° C., in a standard Warburg apparatus using vessels of approximately 15 ml. total capacity ($k_{O_2} = 1.4–1.5$ μl./mm.). The eggs used in these experiments were 90–100% fertilizable following the acid treatment. The settled eggs were diluted to a 20% suspension, on the basis of settled volume, with buffered sea water (0.01 *M* glycyl-glycine, pH 8.0). The suitability of glycyl-glycine as a sea water buffer, and as a medium for the eggs, has been established by Tyler and Horowitz (1937). The main chamber of the vessel contained 2.3 ml. of egg suspension, the center well 0.2 ml. of 10% NaOH, and the side arm 0.5 ml. of the test solution (antiserum, normal serum, hypertonic sea water, or sperm suspension). After the respiratory rate of the eggs in buffered sea water had been established (usually after 40 minutes), the side arms were tipped and the rate of respiration in the test solution was determined. At the end of each experiment, the eggs were examined microscopically.

2) *Strongylocentrotus purpuratus.* During the two preliminary experiments reported in Table II, the procedure followed was similar to that described above, with the following exceptions: (1) The temperature employed was 18° C., the maximum tolerated by these eggs. (2) One ml. of a 20% suspension, in buffered sea water, was exposed to 0.5 ml. of serum, added from the side arm of vessels of ca. 7-ml. total capacity ($k_{O_2} = 0.7–0.8$ μl./mm.). Thus, the ratio of ml. of serum to the final volume in the vessel was increased, in these experiments, from 0.18 to 0.33 ml. serum/ml. (3) In experiment 2, Table II, the egg suspension was exposed to 1 mg% trypsin solution (crystalline, lyophyllized trypsin—Worthington Biochemical Corp., Freehold, N. J.) for 10 minutes prior to suspension in buffered sea water. This treatment prevented the elevation of the fertilization membrane of test eggs inseminated in sea water following washing. The eggs used in both experiments were 95–100% fertilizable after acid treatment. Trypsin treatment reduced this figure to 30% in experiment 2, using the same amount and concentration of sperm suspension for each test insemination.

Results

As can be seen in Table I, all antisera against fertilizin increased the respiratory rate of unfertilized Lytechinus eggs, including those antisera directed against *S. pupuratus* fertilizin. The increase noted in heterologous antisera was somewhat less than that observed using homologous antisera. Normal sera and antiserum

against sperm effected no measureable increase in respiratory rate compared with the rate observed in buffered sea water. The increased respiration in the presence of antisera against fertilizin reached a maximum at 20–40 minutes following the addition of the serum from the side arm. Following this, the rate declined toward the control level, reaching this point in approximately 40 minutes. The rates shown in Table I represent average rates over the entire period of exposure (80 minutes) of the cells to the sera. Maximum rates ranged from 4 to 5 times control rates. Serial dilution of the antiserum (in this case, the antiserum directed against antigen 10 was employed) progressively lowers the value of the maximum observed rate, as indicated in experiment 7, Table I. In experiment 5,

TABLE II

The effect of antisera against fertilizin on the respiratory rate of unfertilized Strongylocentrotus purpuratus eggs

	Aver. rate (μl./min./vessel)	
Experiment No. 1		
Antiserum from rabbit:		
d.	0.15	
e.	0.15	0.15 aver.
f.	0.15	
Pre-injection control serum:		
d.	0.14	
e.	0.14	0.14 aver.
f.	0.14	
Experiment no. 2 (trypsinized eggs)		
Antiserum from rabbit:		
d.	0.19	
e.	0.21	0.20 aver.
f.	0.20	
Pre-injection control serum:		
d.	0.16	
e.	0.17	0.16 aver.
f.	0.16	
Aver. rate, buffered sea water (12 determinations)	0.17 (Range—0.15–0.18)	

dealing with the effects of heterologous antisera directed against the *S. purpuratus* fertilizin antigen, a higher proportion of antiserum relative to the amount of egg suspension was employed in order to duplicate conditions existing during experiments with *S. purpuratus* eggs. The maximum rate observed with heterologous antisera was about three times the control rate. The high (ca. 7 times the control rate) rate observed with the homologous antiserum in this experiment presumably reflects the higher concentration of antibody employed.

As controls, some aliquots of eggs were exposed to sperm suspensions (0.1%) or to hypertonic medium (1 *M* final concentration with respect to NaCl) sea water, added from the side arm of the vessel. The increased respiration observed as a consequence of fertilization or treatment with hypertonic sea water approximates the

increases obtained using homologous antisera against fertilizin (Table I). Increased respiration following exposure to hypertonic medium was first reported by Warburg (1908), and confirmed by Keltch and Clowes (1947). The antiserum against extract of fertilized *Lytechinus pictus* eggs, washed and demembranated prior to homogenization, also proved effective in increasing the rate of respiration of *L. variegatus* eggs (results included in Table I).

Two preliminary experiments using unfertilized *S. purpuratus* eggs yielded less conclusive results. Slight increases in respiratory rate of questionable significance occurred after the addition of homologous antiserum against fertilizin. The increases in rate usually appeared within 30 minutes following addition of the sera, and lasted for 15–20 minutes, after which time the rates returned to the control level. Table II presents average rates during the time (60 minutes) the eggs were exposed to the sera. In experiment 2, the eggs were trypsinized as described above, on the assumption that a barrier exists at the egg surface which prevents combination of antibodies with the necessary sites on the egg. This treatment did not appreciably alter the results obtained during experiment 1. That the antisera against *S. purpuratus* fertilizin contained antibodies capable of increasing respiratory rate can be seen by the ability of these antisera to increase the respiration of *L. variegatus* eggs (Table I). In addition, these antisera were capable of blocking the first cleavage of demembranated fertilized *S. purpuratus* eggs, indicating the presence of antibodies against fertilizin (Tyler and Brookbank, 1956a, 1956b). Furthermore, positive ring precipitin tests were obtained with the homologous antigen. The problem of the failure of these antisera to cause an increase in the rate of respiration of the unfertilized eggs of *S. purpuratus* comparable to that observed using Lytechinus eggs remains unsolved at this writing. Since only two experiments are available, it may well be that future work will resolve the apparent difference between these two species.

Microscopic examination of Lytechinus eggs following these experiments revealed no morphological evidence of activation, excepting, of course, the cases in which the eggs had been inseminated. Samples of Lytechinus eggs treated with immune serum or hypertonic sea water in the manometer vessels were transferred to fresh sea water and observed periodically for 3 to 4 hours. No indications of membrane elevation or cleavage were seen in these eggs, though control eggs inseminated in the manometer vessels elevated fertilization membranes while in the vessels, and developed normally following transfer to fresh sea water. In some instances, Lytechinus eggs exposed to antisera against fertilizin were agglutinated (experiments 2, 3) and some darkening of the cortical region was noted. In most instances, the eggs tended to cytolyze in the antisera against fertilizin after 3–4 hours exposure.

Discussion

The above results indicate that antisera against fertilizin are capable of temporarily elevating the respiratory rate of unfertilized as well as fertilized (Tyler and Brookbank, 1956b) Lytechinus eggs. The time course followed by the inincrease to the maximum rate and the subsequent return to the control rate is similar in both cases. Judging from the increased maximum rate in the presence of a relatively greater amount of antiserum (Table I, experiment 5), it is probably safe to assume that the rate obtained in a given experiment is a function of antibody

concentration, provided the total volume and the number of eggs present remain constant. This is also indicated by experiment 7 (Table I), in which serial dilutions of antiserum against antigen number 10 were tested.

In comparing the results presented in this report with results obtained previously by others, it is apparent that increased respiratory rate in the presence of specific immune serum is seldom encountered, even over a rather wide range of biological material. The report by Sevag and Miller (1948) represents the only instance encountered by this author in which a temporary increase was observed. The increased respiratory rate was found only upon lysis of the *E. typhosa* cells in the presence of active complement, and was not observed when the cells were agglutinated. Complement was not added to the sera employed in the present study, nor were the sera heated to inactivate complement. Thus, the role of complement in the system causing the increased respiration of Lytechinus eggs is not known, though heating antisera against fertilizin to 56° C. for 30 minutes to inactivate C'1 and C'2 does not alter the cleavage blocking property of these antisera (Tyler and Brookbank, 1956a). The unfertilized eggs do not cytolyze during the period of measurement of respiratory rate, and remain intact for 3 to 4 hours following removal from the manometer vessels. After this time, a gradual cytolysis becomes evident. The increased oxygen consumption does not appear, therefore, to be associated with visible cytolytic changes in the egg, since the maximum respiratory rate in the presence of antisera against fertilizin is observed 20–40 minutes following the addition of the antisera from the side arm.

In considering the reports of Perlman (1954, 1957) and Perlman and Perlman (1957) on the parthenogenetic properties of antisera against extracts of unfertilized eggs and against fertilizin, one might be tempted to consider the increased rate of respiration of unfertilized eggs in the presence of antisera against fertilizin, or in hypertonic media, to be indicative of activation. This conclusion does not seem warranted by the data presented in this report. No morphological evidence of activation was encountered during the experiments, and one might more properly consider the increased respiration of eggs so treated to be analogous to the increases obtained in the presence of nitro-phenols (Clowes and Krahl, 1934, 1936; Krahl, 1950), methylene blue (Ballentine, 1940) and other substances which are not considered parthenogenetic. With regard to the failure to observe activation of eggs exposed to hypertonic sea water, it should be recalled that the time of exposure of unfertilized eggs to the proper hyper- or hypotonic medium is critical (Harvey, 1940). Therefore, failure to observe morphological signs of activation under the conditions prevailing in the manometer vessels is not surprising. In addition, a wide range of hyper- or hypotonic media are capable of eliciting increased respiration of the unfertilized eggs without effecting activation. The extent of the increases obtained depends on the degree of hyper- or hypotonicity employed, in much the same way as the extent of increases obtained with antisera against fertilizin depends on the amount of antibody present (Table III).

In conclusion, it seems appropriate to consider the purity of the fertilizin antigens used for injection. Special precaution was taken in the preparation of the *S. purpuratus* fertilizin antigen, and antigen number 10 (*L. variegatus* fertilizin), to insure minimum contamination with material from the eggs. These antigens were purified according to methods designed to yield electrophoretically homogene-

ous fertilizin, and were injected in exceedingly small amounts. The initial removal of fertilizin from the eggs was carried out at pH 3.5. Eggs treated at this pH for 2–3 minutes and returned to pH 8 develop normally, ruling against damage to the eggs by this degree of acidity. Antigen number 4 (*L. variegatus* fertilizin) was obtained in the same manner as number 10, except that the step involving precipitation of the antigen with ethanol was omitted. Results obtained using antisera against antigen number 10 paralleled those obtained using antisera against antigen number 4 completely. Apparently antisera against purified fertilizin are capable of temporarily raising the respiratory rate of the unfertilized egg. Results obtained using antiserum against extract of fertilized eggs parallel those obtained using antisera against fertilizin, since this antiserum also increased the rate of respiration of the unfertilized eggs. Since antibodies most probably cause their effects through combination with fertilizin at the egg surface, this last mentioned result seems to indicate the presence of fertilizin haptens in the fertilized-egg antigen. The most

TABLE III

The effect of varying degrees of hyper- and hypotonicity on the respiration of unfertilized Lytechinus variegatus eggs. Conditions the same as those prevailing for the experiments in Table I. (Hypertonicity in terms of excess NaCl, hypotoncity in terms of added distilled water)

Tonicity	Aver. rate µl./min./vessel
Experiment 1—hypertonic media	
2.9 × sea water	0.97
1.85 × sea water	0.55
Sea water	0.20
Experiment 2—hypotonic media	
0.84 × sea water	0.50
0.75 × sea water	0.58
0.66 × sea water	0.67
Sea water	0.28

probable location of these fertilizin haptens is the hyaline layer (ectoplasmic layer) of the fertilized egg, as proposed by Tyler and Brookbank (1956a).

SUMMARY

1. Homologous antisera against purified fertilizin, and against extract of fertilized eggs (of *Lytechinus pictus*) have been shown to temporarily increase the respiratory rate of the unfertilized eggs of *L. variegatus*. Parallel experiments employing antisera against fertilizin of *Strongylocentrotus purpuratus* and unfertilized *S. purpuratus* eggs yielded essentially negative results. Further experimentation is necessary before this apparent difference between the responses of the eggs of these two species to antisera against fertilizin can be resolved.

2. Antisera against fertilizin of *S. purpuratus* were effective in increasing the respiratory rate of unfertilized *L. variegatus* eggs, indicating the presence of antibody capable of effecting increased respiration.

3. Normal sera and antiserum against sperm were without measurable effect on the respiratory rate of *L. variegatus* eggs.

4. None of the eggs treated with antisera against fertilizin showed morphological evidence of activation.

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A BICOLORED GYNANDROMORPH OF THE LOBSTER, HOMARUS AMERICANUS

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Lobsters with sharply defined, bilateral color differentiation have been described by several authors. Herrick (1896) mentioned the following variations of this pattern in both the American and the European lobster: half normal color and half light sky blue; half normal and half pale red; half greenish black and half light orange; half blue and half white; and half light yellow and half bright red. Schaanning (1929) gave a color figure of a European lobster that was light red and dark blue. Templeman (1948) added records of two more bicolored American lobsters, one normal and red, the other whitish red and purplish blue. Such color variants have occasionally been referred to as gynandromorphs or hermaphrodites, but there is no evidence that any of the previously recorded bicolored specimens were also bisexual.

Only two cases of possibly complete hermaphroditism have been recorded heretofore for *Homarus*. Nicholls (1730) described and figured a specimen of the European lobster, *H. gammarus*, received from Newgate-Market, London, that displayed all of the external and internal female characters on the right side and all of the male structures on the left. Halkett (1919) collected a specimen of *H. americanus* at Bay View, Pictou County, Nova Scotia, November 1917 "which was absolutely male on the left side and absolutely female on the right side"; this specimen was sent to Queen's University, Kingston, Ontario, but apparently no complete description of it has been published. Gordon (1957) described a specimen of *H. gammarus* from off Seahouses, Northumberland, that had all of the characters of a perfect gynandromorph—female on the right side, male on the left—except that there was no male opening on the left fifth pereiopod but, instead, an imperforate indication of an opening on the coxa of the left third pereiopod; this specimen was not dissected, but Dr. Gordon suggests that "it probably has a normal ovary on the right side and part ovary, part testis on the left side—or a testis with ova in the anterior position." Herrmann (1890) discovered that eggs are occasionally developed during spermatogenesis in the lobster testis but he gave no indication that this was associated with any unusual external characteristics. Finally, Ridewood (1909) recorded an ovigerous specimen of *H. gammarus*, presumably from off the Orkney Islands, that had genital openings on the third right pereiopod and on the fourth and fifth left pereiopods, but dissection disclosed only a normal paired ovary with apparently three oviducts, two of them on the left side leading to the abnormally placed openings.

The specimen described below (U. S. Nat. Mus. Cat. No. 102241) seems to be the first lobster to be recorded in which a color anomaly was associated with

gynandromorphism. The specimen was alive when presented to the Fish and Wildlife Service at Woods Hole, Massachusetts, during the summer of 1954 by a dealer operating between Boston and Cape Cod. Its place of origin is unfortunately unknown; it probably came from Massachusetts Bay but it could have been shipped from New Hampshire, Maine, or even Canada. It died while being photographed by John P. Wise, who offered it to the junior author for description. After remaining in a freezer for about six months, it was transferred to formalin for dissection; the dissected portion is stored in ethyl alcohol, and the carapace, abdominal tergites, tail fan, and chelipeds have been dried.

The specimen was about 10 inches (25 cm.) long from the tip of the rostrum to the end of the telson. The carapace measures 86 mm. from the eye socket to the hind margin. To the left of the midline of the animal, as well as on most of the gastric and hepatic regions on the right side, the ground color was orange, mottled and spotted with dark, greenish brown. The right side, posterior to the mesogastric and hepatic regions, was similarly spotted and mottled but in shades of blue over a lighter blue ground color, reminiscent of the colors of willow-pattern china. The color pattern is indicated in Figure 1. The spotting and mottling pattern seems to be continuous across the midline; only the color is different. The color transparency made from the living animal suggests that blue pigment was largely lacking on the left side, and red, yellow, and possibly black pigments were missing on the right side, although there is a greenish cast to the dark mottling on the left and a pinkish tinge in some of the light blue areas on the right.

The left, crusher cheliped was colored like the left side of the body for the most part, but the color photograph suggests that there were blue patches at the outer, distal end of the merus, on the dorsal surface of the carpus, on the thick portion of the hand, near the base of the fixed finger, and on the base of the dactyl. The spines on this cheliped were bright red. The right, cutting cheliped was blue, with a tinge of brown near the base of the fixed finger, and the spines were almost pure white. The other pereiopods on the left side were orange with greenish brown shadings similar to those on the adjacent portion of the carapace. Those on the right side were very light, pinkish blue with darker blue shadings. The left uropods were orange with greenish brown margins, and those on the right, pale, pinkish blue with dark blue marginal bands. The fringe of setae on the left uropods and on the telson to the left of the midline were reddish orange, those on the right side, yellowish orange.

As in the three previously described gynandromorph lobsters, the specimen displayed female characters on the right side and male structures on the left. The body was skewed to the right anteriorly and to the left posteriorly as shown in Figure 1. This was almost certainly a result of the differential growth of male and female lobsters. Templeman (1944) stated: "At all commercial sizes the relative length of the carapace is less in the female than in the male. It remains constant when the lobsters are small, but shows a definite and progressive increase for those localities for which large lobsters were available for measurement. . . . The ratio of the greatest width of the carapace to total length is in the smaller relatively immature lobsters approximately the same for both males and females, while in the larger animals it increases with size and more so in the male than in the female." The skewing of the axis and the more swollen left (male) side

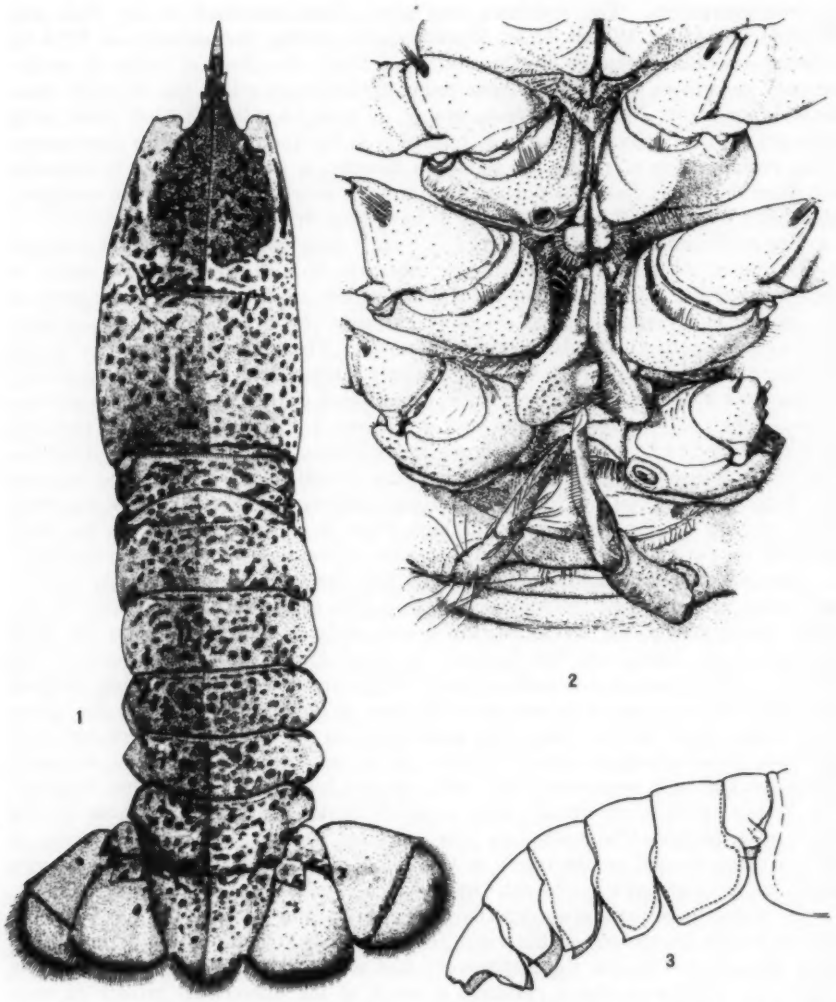

FIGURE 1. Dorsal surface of body of gynandromorph of *Homarus americanus* showing asymmetry and color pattern. Drawn from color transparency of living animal.

FIGURE 2. Ventral surface showing opening of oviduct on coxa of third right pereiopod and of vas deferens on fifth left pereiopod, asymmetrical thelycum, and characteristic female and male first pleopods.

FIGURE 3. Abdominal somites viewed from the right (female) side. The left (male) pleura are shown as if viewed from the inside.

of the carapace are therefore readily understandable. The reverse skewing of the abdomen probably also resulted from this differential growth, accentuated by the proportionately larger abdominal pleura in the female, as shown in Figure 3. The greater apparent length of the left (male) pleura of the fourth, fifth, and sixth somites in this figure is misleading and is caused by the fact that the left (male) pleura curve downward nearly vertically, whereas those on the right (female) side extend obliquely outward, as shown in Figure 1. The distortion is less striking than in Gordon's larger (11-inch) specimen of *H. gammarus* and in Nicholls' specimen of the same species, the size of which was not given. This might be expected from Templeman's (1944) findings that the differential growth of males and females becomes progressively more marked with age.

The most noticeable disparity in our specimen is found on the ventral surface. As shown in Figure 2, there is a female opening on the coxa of the right third pereiopod and a male opening on the left fifth pereiopod, which is represented only by the coxa and basis. The thelycum is distinctly asymmetrical, the right (female) part being broad and nearly bare and the left (male) part narrower and provided with long hairs. Neither element of this structure corresponds exactly with its form in normal males and females, but the similarity to the conditions in the appropriate sexes is more than superficial. Even the median plate extending forward from the last thoracic sternite is modified as would be expected: the right portion is longer and acute, like half of a typical female plate, and the left portion is shorter and rounded as in the male.

The first right pleopod is typically female, flexible and long-haired, while the left one is a rigid male intromittent organ. There is a well-developed appendix masculina on the endopod of the left second pleopod, but none on the right member of this pair. The second, third, fourth, and fifth pleopods on the left (male) side are 37.5, 35.3, 33.5, and 29.0 mm. long, respectively, from the basal articulation to the end of the endopod. Those on the right (female) side have corresponding lengths of 37.2, 38.5, 38.0, and 33.3 mm. These figures agree remarkably well with the proportionate lengths of these appendages in normal males and females, as determined by Templeman (1944): "In the male the swimmerets (including protopodite and endopodite) on the second abdominal segment are the longest and in the female those on the third and fourth are the longest and approximately equal. The second swimmerets are not greatly different in length in males and females. . . ." The sternal spines of the gynandromorph are about 2.5 mm. long on the second, third, and fourth abdominal somites and about 2.0 mm. long on the fifth somite. Data given by Templeman (1944) for New Brunswick specimens indicate that the average length of the spine on the second somite in specimens of comparable size is about 3.75 mm. in males and 0.5 mm. in females, and the spine on the fifth somite is about 2.5 mm. long in males and 0.5 mm. in females. The spines in the gynandromorph therefore seem to be intermediate in size, perhaps more nearly approaching the male than the female condition.

Careful removal of the carapace and abdominal tergites of the specimen disclosed a well-developed ovary filled with maturing eggs on the right side and a normal testis on the left, as shown in Figure 4. An oviduct led from beneath the ovary to the opening on the right third pereiopod and a typical vas deferens connected the testis with the left fifth pereiopod. A few of the eggs in the ovary appeared slightly discolored. Herrick (1911) stated that the presence of orange flecks

in the ovary, representing degenerating eggs that were not shed, is conclusive evidence that a lobster has already spawned at least once, but the spots in our specimen were not sufficiently distinct to permit an unequivocal determination that spawning had occurred. As can be seen in Figure 4, a lobe of the ovary, probably representing the connection between the two halves of the organ in a normal female, was found just anterior to the heart. At this point the testis was interrupted and the two parts of it were continuous with the intermediate portion of the ovary. It appeared that the two portions of the testis were differentiated parts of a single organ. There is no doubt that the testis was functional, for sections showed active spermatogenesis. Normal, fully formed spermatozoa were extracted from the vas deferens.

It is, of course, impossible to determine whether this specimen could have functioned reproductively as either or both a male and a female lobster. Viable spermatozoa and eggs were probably produced, but the unpaired intromittent organ and the deformed thelycum might have prevented successful copulation with normal males and females. The specimen must have been nearly or quite mature. As

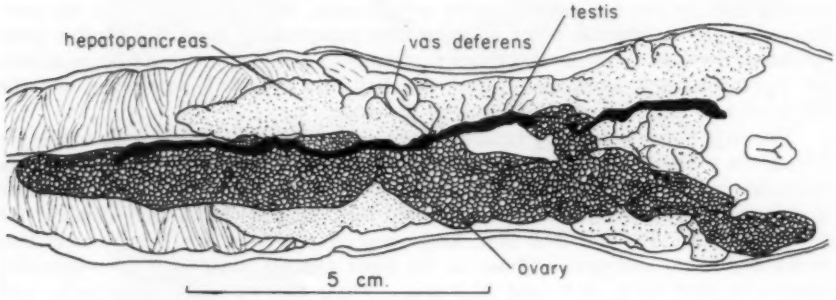

FIGURE 4. Dissection of lobster gynandromorph after removal of heart and stomach showing well-developed ovary on right side and testis and vas deferens on left.

mentioned above, we were unable to determine whether or not it had spawned. If it was caught off the Massachusetts coast, it was probably six or seven years old according to Herrick (1911). Herrick (1896) also maintained that "very few lobsters under 9 inches in length have external eggs, while only few have attained the length of 10½ inches without having them." Templeman and Tibbo (1945) concluded from the examination of New Brunswick specimens that the length of males at sexual maturity is at least 5 cm. less than that of females. One can hardly assume, however, that the size of a gynandromorph is directly comparable with that of a normal individual of either sex. Female lobsters probably grow more slowly than males (Herrick, 1911), and one might therefore expect the present specimen to be smaller than a normal male and larger than a normal female of the same age, but the growth rates of abnormally bisexual crustaceans may be complicated by hormonal or other factors.

We wish to thank John P. Wise for calling our attention to this unusual specimen. We also wish to acknowledge the assistance of members of the staff of the

Division of Marine Invertebrates, U. S. National Museum, during the preparation of the paper. Special thanks are due Charles E. Cutress of that staff for the histological preparation and study of the reproductive organs of the specimen.

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RE-EXAMINATION OF AN INHIBITOR OF REGENERATION IN TUBULARIA

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In the marine hydroid *Tubularia* the presence of hydranth structures has been thought to prevent the development of new hydranths in nearby stem tissue. Two preparations have been made from adult hydranths which inhibited the regeneration of new hydranths on isolated stem segments. One of these (***inhibitor water***, Rose and Rose, 1941) was made by agitating adult hydranths in aerated sea water for from 12 to 24 hours, while the other (***hydranth extract***, Tardent, 1955) was found in the supernatants of homogenates of adult hydranths. These inhibitors of regeneration were specific to hydranth tissue in that they were not obtained when stems were treated in the same manner. They have been compared (Tweedell, 1958) and found to differ in a number of properties. The regeneration-inhibiting substances in inhibitor water have been considered by a number of authors to represent the substances normally responsible for physiological dominance in *Tubularia*, and inhibitor water has been employed by Steinberg (1954) in an experiment to indicate the mechanism of physiological dominance.

In the present investigation it was found that active inhibitor water could not be prepared in the absence of bacterial growth, and as a consequence a re-examination of this inhibitor was undertaken.

Materials and Methods

Freshly-collected *Tubularia crocea*, provided by the Supply Department of the Marine Biological Laboratory, was used in all experiments. Sea water was filtered through paper shortly before use.

In preparing inhibitor water, an attempt was made to use methods comparable to those used by previous workers (*cf.* Tweedell, 1958). Populations of adult hydranths with 5 mm. of stem attached were isolated and washed thoroughly, and then aerated in sea water for 24 hours at 17–22° C. After aeration, the hydranths and debris were removed by filtration and the preparation was tested for its effect on regeneration.

The bacterial population was estimated subjectively in early experiments by turbidity and microscopical examination, and in later experiments was determined using a Petroff-Hauser bacteria-counting slide. The bacterial population of filtered sea water was found to be approximately 10^5 per ml., which is too low for accurate estimation with a counting slide. It was assumed that no bacterial proliferation had occurred during the preparation of any given solution if the bacterial population did not exceed this order of magnitude. It should be cautioned that if mature male hydranths are used to prepare inhibitor water, turbidity may in part result from the release of large numbers of sperm into the water.

When it was desired to remove bacteria, the preparations were filtered through an HA millipore filter (Millipore Filter Corp., Watertown, Mass.) or centrifuged for 5 minutes at 30,000 g. Bacterial growth was prevented by the addition of antibiotics. Penicillin and streptomycin were used at 100–125 μg./ml.; sulfadiazine was used at about 0.001 per cent (or saturation in sea water). At these concentrations, and in the cases of penicillin and streptomycin even at four-fold higher concentrations, the antibiotics did not have any significant effect on the rate or course of regeneration.

The solutions to be described were tested immediately after preparation for their effect on the regeneration of freshly-cut, 7-mm. stem segments. Virtually all of the stems in control groups regenerated, although as is usual with *Tubularia* there was a considerable variation in the rate, even within a single group. A preparation was considered to have inhibited regeneration if, during the time required for the complete regeneration of the controls (emergence), all or a significant fraction of the experimental group either disintegrated or healed but did not begin regeneration. Stems in inhibitor water which regenerated were usually but not always retarded.

Results

Populations of 1.5–2 hydranths per ml. aerated in sea water regularly produced an inhibitor water which completely prevented regeneration. Occasional batches of inhibitor water prepared at these or lower hydranth densities were inactive, while populations of more than two hydranths per ml. usually gave a preparation which caused disintegration of the stem tissue. However, considerable variability was found in the activity of preparations made at the same hydranth densities and under the same conditions (temperature, time, etc.), suggesting that some factor other than those controlled was involved.

A number of observations suggested that the activity of inhibitor water was due to bacterial growth. Preparations became quite turbid during the course of aeration, and the condition of the hydranths deteriorated rapidly. The solution developed a putrid odor. Hydranths killed by exposure to 30° C. for 15 minutes rapidly disintegrated, but nevertheless produced active inhibitor water. When active preparations were examined microscopically, a large, heterogeneous population of bacteria was found. Removal of these bacteria often resulted in a reduction but never in an elimination of the inhibitory activity of a preparation.

An estimate of the amount of bacterial growth which occurs in inhibitor water preparations was obtained by preparing a series of 5 inhibitor waters at a density of 1.5 hydranths per ml. and making counts with a bacteria counting slide at the beginning and end of aeration. In this series, the bacterial density increased during aeration from about 3×10^5 bacteria per ml. to about 10^8 bacteria per ml. Bacteria were removed by centrifugation and each preparation tested for its effect on the regeneration of 10 stems. The results are given in Table I. The increase in bacterial number represents a minimum of 9 generations of bacterial growth. It should be noted, however, that counts made at the beginning of aeration do not include bacteria which are present in the hydranths and are released into the water during aeration as the hydranths disintegrate.

In order to determine whether or not hydranths could produce active inhibitor water in the absence of bacterial growth, hydranths were agitated in sea water

containing antibiotics at concentrations sufficient to maintain bacteriostasis. The results of three of the experiments with penicillin and streptomycin are given in Table II. In the first two experiments shown (A and B), the amount of bacterial growth was estimated subjectively. No detectable bacterial growth occurred in any of the preparations in experiment A, and in spite of the fact that the hydranth density was more than twice that necessary to produce complete inhibition in the absence of antibiotics, the stems in both experimental groups all regenerated at the same rate as the control stems. In experiment B, the hydranth density was over four times that necessary to produce complete inhibition without antibiotics. The preparation agitated at this hydranth density with antibiotics (B4) produced some delay in the rate of regeneration. This delay may have been due to the cytolysis of some of the hydranth tissue releasing the same substances present in hydranth extracts (see discussion).

These experiments indicate that in the presence of penicillin and streptomycin in concentrations which suppress bacterial growth, inhibitor water cannot be collected. However, it may be argued (Tweedell, 1958) that the antibiotics used either prevent the hydranths from producing an inhibitor of regeneration or destroy this inhibitor as it is produced. Three observations appear to exclude these

TABLE I

The number of bacteria present in five similar preparations of inhibitor water and the effect of these preparations on regeneration. Observations were made at intervals for 4.3 days

Preparation	Bacteria/ml. $\times 10^3$	Effect of preparation on stems
Control	0.0	10/10 emerged within 2.4 days
1	2.3	10/10 did not begin regeneration
2	3.3	10/10 did not begin regeneration
3	3.7	10/10 disintegrated within 3.6 days
4	5.5	10/10 disintegrated within 1.4 days
5	7.4	10/10 disintegrated within 1.0 day

alternatives. (1) That penicillin and streptomycin do not destroy the inhibitors was demonstrated by experiments in which bacteria were removed by centrifugation and these antibiotics added to inhibitor water after preparation. In one such experiment, there was no measurable reduction in the activity of the preparation when antibiotics were added (Table II, C3); in another (not listed) there was a slight reduction but not an elimination of the inhibition produced by the preparation (similar to that observed in other preparations when they were sterile filtered). (2) Particularly important are three experiments with penicillin and streptomycin and one with sulfadiazine in which bacterial growth occurred in the preparations even though antibiotic was added at the beginning of aeration. Presumably bacteria resistant to the antibiotics used developed in these preparations. In these cases, the preparations inhibited regeneration in proportion to the amount of bacterial growth which occurred in them (*e.g.*, Table II, C5, 6), showing that, in spite of the antibiotics, if bacterial growth occurred, an active regeneration inhibitor was produced. (3) Three antibiotics—penicillin, streptomycin and sulfadiazine—differing greatly in chemical structure and presumed mode of action, were used alone or in pairs to maintain bacteriostasis. Regardless of which antibiotic was used, if bacterial growth was prevented the preparation failed to inhibit regeneration.

To see if hydranths were a necessary component of the system, experiments were done in which bacterial growth was allowed to occur in sea water in the absence of hydranths. Dilute proteose-peptone solutions in sea water, aerated for 24 hours, and then sterilized by millipore filtration followed by the addition of antibiotic, were potent inhibitors of regeneration, while control solutions in which

TABLE II

Selected experiments which illustrate the activity of inhibitor water prepared with penicillin and streptomycin. Bacterial density was either estimated (number represented by pluses in the table) or counted directly using a bacteria counting slide (represented by number per ml.). Abbreviations: pen., penicillin; strep., streptomycin

Experiment	Components added to sea water	Bacteria per ml.	Stems regenerated vs. total	Mean time of emergence in days
A1	Pen. and strep.	—	10/10	2.5
2	4 hydranths/ml. + pen. and strep.	—	10/10	2.6
3	4 hydranths/ml. + pen. and strep.	—	10/10	2.5
B1	None	—	10/10	2.3
2	8 hydranths/ml.	+++	0/10	—
3	Pen. and strep.	—	10/10	2.4
4	8 hydranths/ml. + pen. and strep.	—	10/10	3.1
C1	None	ca. 10^6	10/10	2.4
2	2 hydranths/ml.	5×10^8	0/10	—
3	2 hydranths/ml., pen. and strep. added after aeration*		0/10	—
4	Pen. and strep.	$< 10^5$	10/10	2.3
5	2 hydranths/ml. + pen.	2×10^7	5/10	4.8
6	2 hydranths/ml. + strep.	1×10^8	2/10	2.3
7	0.1% proteose peptone + pen. and strep.	ca. 10^5	10/10	3.1
8	0.1% proteose peptone, pen. and strep. added after aeration	3×10^8	0/10	—

* Penicillin and streptomycin were added to a portion of solution C2.

bacterial growth was prevented by the addition of antibiotic at the beginning of aeration, at most, slightly retarded regeneration (*e.g.*, Table II, C7, 8; compare C1, 2).

To make certain that the inhibition produced as a result of bacterial growth was not dependent on the presence of specific bacteria, preparations were made

using *Escherichia coli*. Cultures were grown in a minimal medium (Davis and Mingioli, 1950) from a small inoculum to 10^9 cells per ml. The bacteria were removed by centrifugation, and the used medium diluted 1:5 in sea water containing antibiotic. Such a preparation completely inhibited regeneration, while control stems placed in a 1:5 dilution of sterile minimal medium with antibiotic regenerated normally.

From these data it is clear that the activity of inhibitor water can be explained on the basis of the bacterial growth which occurs in the medium, and that no other inhibitors can be collected when bacteriostasis is maintained with antibiotics.

TABLE III

Summary of all experiments which indicate that inhibitor water is a by-product of bacterial growth. Refer to the text for explanations of each experiment. Abbreviations: pen., penicillin; strep., streptomycin; sulfa., sulfadiazine

Components added to sea water before aeration	Number of experiments	Bacterial growth	Inhibition of regeneration
None	17	−	−
Hydranths	17	+	+
Hydranths*	6	+	+
Hydranths**	2	+	+
Heat-killed hydranths	4	+	+
Pen., strep., or sulfa.	14	−	−
Hydranths + pen., strep., or both pen. and strep.	7	−	−
	3	+	+
Hydranths + sulfa.	2	−	−
	1	+	+
Stem lengths	2	−	−
Proteose peptone, pen. and strep.	3	−	−
Proteose peptone**	4	+	+

* Preparation sterile filtered after aeration.

** Preparation centrifuged after aeration, penicillin and streptomycin added to the supernatant.

As an argument for the specific role of hydranth structures in producing inhibitor water it has been noted that a population of stems, aerated in sea water, does not produce an inhibitor (Tweedell, 1958). After cutting, the ends of a stem rapidly heal and secrete a thin layer of perisarc, so that very soon a cut stem is entirely covered with chitin. Since no tissue is exposed, a preparation of stems could not be expected to be a good medium for bacterial growth, and this might be

the reason why no inhibitor was produced. Experiments were done in which populations of clean stems were cut, washed, and aerated in sea water. Such preparations did not support the growth of significant numbers of bacteria, and, when tested on stems, permitted regeneration at the same rate as the controls.

A summary of the experiments which have been described, together with the number of cases of each type, is presented in Table III. Cases in which very slight bacterial growth occurred in the preparations or in which the preparations only produced a slight delay in regeneration (such as case B4, Table II) are recorded as negative (−) in the table; only definite cases of bacterial growth or regeneration inhibition are recorded as positive (+). As the table indicates, the inhibition of regeneration was always correlated with the growth of bacteria.

It is pertinent to mention certain experiments done with the regeneration inhibitor found in *Tubularia* hydranth extracts. Such extracts were prepared by homogenizing a population of adult hydranths and collecting the supernatant, as described by Tardent (1955) and Tweedell (1958). It was found that the inhibition of regeneration produced by such extracts was not a result of bacterial growth, in that when penicillin and streptomycin were added to the extracts to maintain bacteriostasis the activity of the extracts was not affected in terms of the proportion of stems inhibited by a given dilution of extract. It was found, however, that in contrast to the original report of Tardent (1955), the inhibition produced by *Tubularia* tissue extracts was not specific to hydranth tissue. The supernatant of homogenates from equivalent quantities of stem tissue also suppressed the regeneration of stems. Tardent (personal communication) has obtained the same result recently with *Tubularia larynx*. Preliminary comparisons on a wet weight basis indicate that hydranth tissue is about twice as active a source of inhibitor as stem tissue. The lack of specificity of this inhibitor makes it impossible, however, in the absence of further data, to adequately evaluate the normal physiological role of the substances involved.

Discussion

The results of the experiments with inhibitor water may be summarized as follows. (1) Hydranths agitated in sea water produce bacterial growth and inhibitors of regeneration. (2) If bacterial growth is suppressed with antibiotics, regeneration inhibitors cannot be collected. (3)If antibotics are added at the beginning of aeration but bacterial growth is not prevented, inhibitors can be collected. (4) Bacterial growth in the absence of hydranths produces regeneration inhibitors. These results, together with the appropriate controls, demonstrate that inhibitor water as prepared in these experiments is a by-product of bacterial growth for which the hydranths serve as inoculum and nutrient source. The results, however, should not be taken to indicate that hydranths cannot produce any inhibitors of regeneration, but rather that inhibitor water prepared as described by previous workers contained no inhibitors which could not be accounted for as the products of bacterial rather than hydranth metabolism.

If hydranths are agitated with antibiotics at densities several-fold higher than those used to prepare inhibitor water (*cf.* Tweedell, 1958), occasionally such preparations (*e.g.*, Table II, B4) retard regeneration even though bacteriostasis has been maintained with antibiotics. It is interesting to note that in such cases

bulbous outgrowths appear at one or both ends of many of the stems. These outgrowths are similar to those found in stems placed in *Tubularia* tissue extracts (Tweedell, 1958; author's unpublished observations), suggesting that the cytolysis of some of the hydranth tissue has released the substances found in hydranth extract into the water.

Since this manuscript was originally submitted for publication, a paper by Tweedell (1958) has appeared in which the results described in the present paper are discussed. The results of this work were presented incompletely by Tweedell; the results as presented here answer the objections raised in his discussion. In particular, the possibility that the antibiotics used had significant effects other than that of maintaining bacteriostasis has been excluded by the results described above.

Tweedell notes that although bacteria were removed from some of his preparations by sterile filtration the preparations still inhibited regeneration. It is clear from the present work that it is not the bacteria themselves, but rather the metabolites they release into the medium, which are primarily responsible for the activity of inhibitor water. Removal of the bacteria from inhibitor water or proteose-peptone solutions after aeration by filtration or centrifugation, or the addition of penicillin and streptomycin to such preparations, in some cases reduced the inhibitory activity of the preparation but in no case eliminated it.

Summary

1. Rose and Rose (1941) found that adult *Tubularia* hydranths agitated in sea water produced a solution, inhibitor water, which prevented regeneration. They and subsequent workers have ascribed to this inhibitor a role in normal physiological dominance. In the present investigation it has been found that considerable bacterial growth occurs in the solution during the preparation of inhibitor water by the usual methods, and that when antibiotics have been added to maintain bacteriostasis no inhibitor can be collected. Experiments have excluded the possibilities that the antibiotics used are preventing the production of the inhibitor or destroying it as it is produced. It has been shown that metabolites produced by bacterial growth in the absence of hydranths inhibit regeneration.

2. These data lead to the conclusion that inhibitor water represents the by-products of bacterial growth for which the hydranths serve as source of inoculum and as nutritive medium.

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STUDIES ON THE STRUCTURE AND PHYSIOLOGY OF THE FLIGHT MUSCLES OF BIRDS. 4. OBSERVATIONS ON THE FIBER ARCHITECTURE OF THE PECTORALIS MAJOR MUSCLE OF THE PIGEON

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Denny-Brown (1929), studying the red and white muscles of vertebrates, made some observations on the "light" and "dark" muscle fibers in the breast muscle of the pigeon. The later works on these two types of fibers have been reviewed by George and Naik (1957). More recently, George and Naik (1958a, 1958b) have shown that the red narrow fibers are rich in fat and mitochondria in sharp contrast to the white, broad, glycogen-loaded fibers, which contain only a negligible amount of fat and mitochondria. George and Scaria (1958a) histochemically demonstrated higher lipase activity in the red narrow fibers. The Krebs' cycle enzymes, too, seem to be localized in the narrow fibers (George and Scaria, 1958b). These findings have stimulated considerable interest and called for a basic understanding of the nature and disposition of the fiber components of this muscle as a whole. The present study, therefore, is an attempt to provide a comprehensive picture of the pattern of fiber distribution and the nature of the metabolite load in the different regions of the muscle.

Materials and Methods

In order to obtain uniformly well developed *pectoralis major* muscle, only fully grown wild pigeons, either shot or trapped from a single locality, were used throughout for the present study.

Mapping the distribution of the two types of fibers in the muscle

Due to the bipectinate arrangement of the fasciculi, it was found convenient to divide the muscle into twelve regions, each one extending to 10 mm. in length along a hypothetical line, drawn midway between the origin of the muscle fasciculi and the centrally placed tendon (as shown in Fig. 1). From each of these regions at the level of the aforesaid line, fresh frozen transverse sections were cut on a freezing microtome. Subsequently the sections were treated in the following manner. Transferring a fresh frozen section into distilled water or even saline or isotonic sucrose solution resulted in uneven curling up of the section. Again, the size of the muscle piece handled being large, some difficulties which were encountered in the beginning in obtaining a good entire section, were completely avoided by transferring the section directly into chilled 50% glycerol and mounting it on a microslide in the glycerol solution. In the preparations thus made the

arrangement of the fibers in the section, however large, was faithfully maintained with no distortions taking place. The glycerol-impregnated sections were thus found to be ideal to manipulate. Moreover, the sections left in glycerol solution and maintained at 0° C. can remain for more than a week without any perceptible defect and thus could be utilized for future observations.

The desired region of the mounted section was projected on the screen of a microphotographic camera at a magnification of 47 × and the photographic printing paper exposed directly to the image. "Normal" bromide papers were found

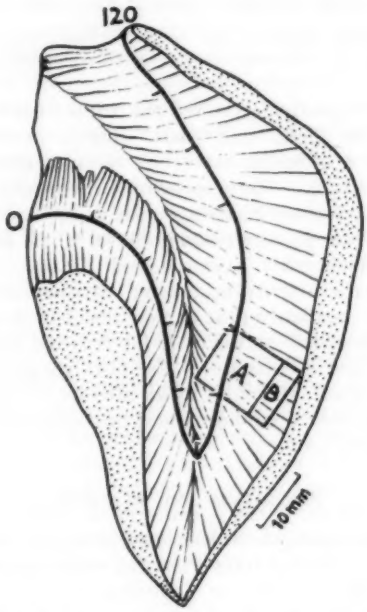

FIGURE 1. Dorsal view of the *pectoralis major* muscle of the pigeon showing the hypothetical lines 0–120 along which the distribution of broad fibers is recorded in Figure 2. The squares A and B indicate the regions of the muscle used for studying the variation in metabolite load and the structure at different depths of the muscle.

suitable. Using the sliding vernier on the stage of the microscope, continuous photographic records of the distribution of the broad fibers were made (Fig. 5). From such records by the method of random sampling, the mean value of the number of broad fibers per square mm. was determined for every mm. depth of the muscle. A survey of all the twelve regions was thus completed and a graph plotted illustrating the continuous distribution of broad fibers per square mm. at the distance of every 5 mm. along the line 0–120 (Fig. 1). The lines demarcating the areas containing 30–50, 50–70, 70–90, 90–100, 100–120 and 120–140 and 120–150 broad fibers per square mm. were drawn. The entire procedure was

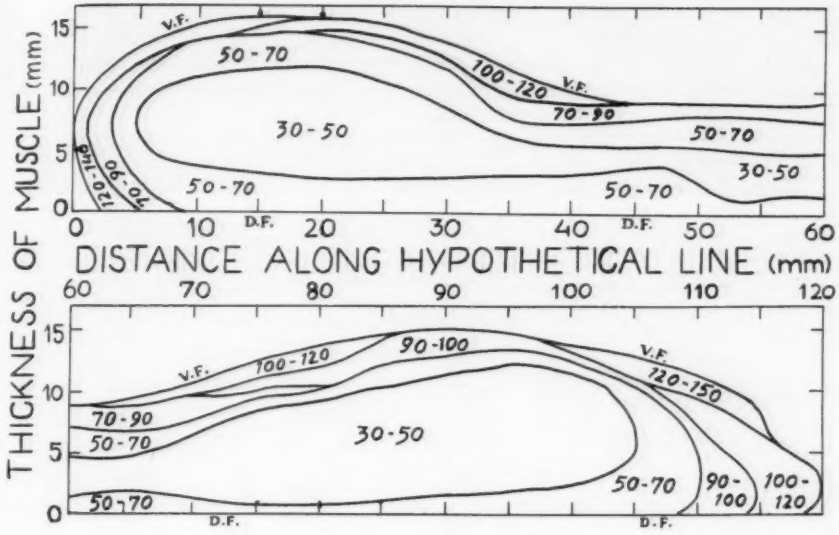

FIGURE 2. Cross-sectional view of the *pectoralis major* along the line 0–120 drawn in Figure 1. The figures in the chart show the number of broad fibers per square mm. D.F., dorsal face of the muscle; V.F., ventral face of the muscle.

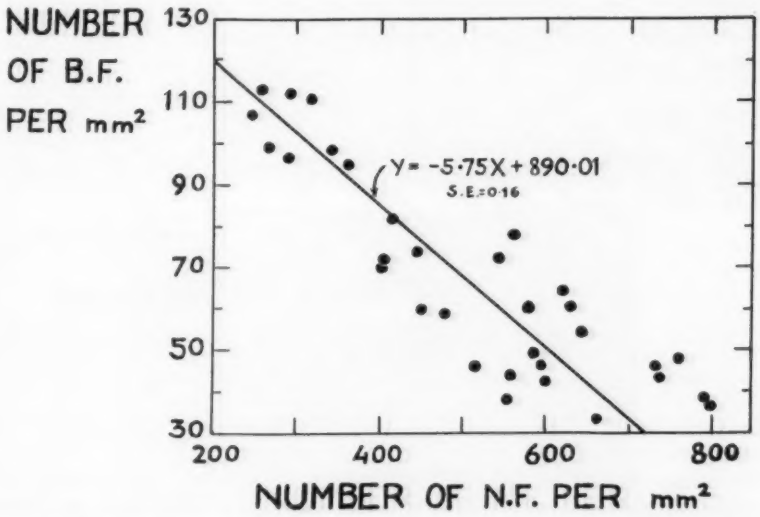

FIGURE 3. Relation between the number of broad fibers and the number of narrow fibers per square mm. of transverse section of the muscle.

repeated on the *pectoralis* of three pigeons. The results obtained are summarized in a graphical representation as shown in Figure 2. Since the individual variations in the *pectoralis* of different pigeons are considerable, the lines demarcating different areas in the figure are not claimed to be absolute, but they do show the

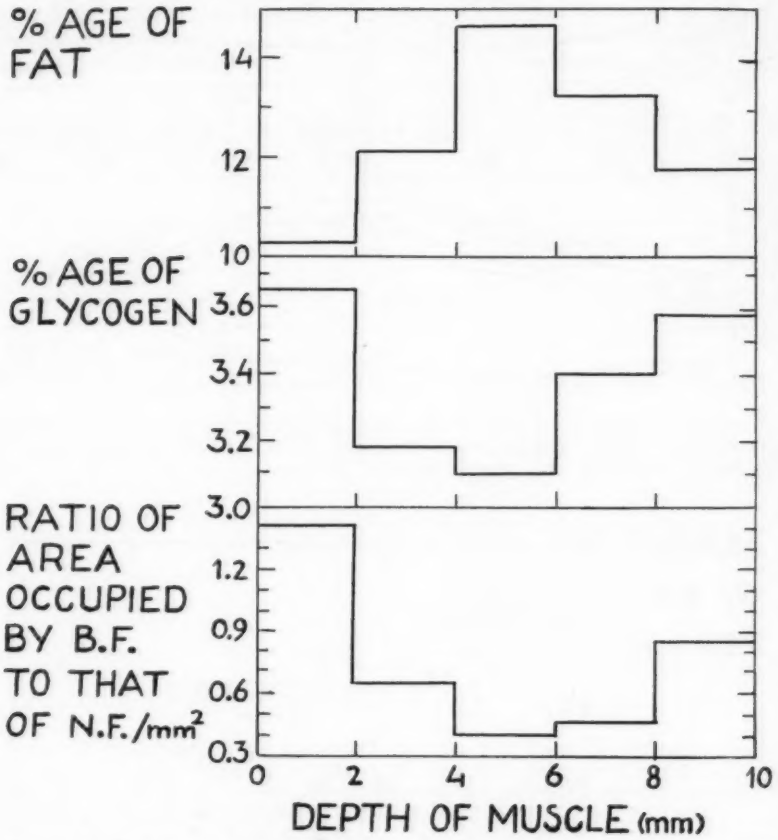

FIGURE 4. Variation in the percentage of glycogen and fat, in relation to the ratio of the area occupied by the broad fibers to that of the narrow fibers per square mm., at different depths of the muscle. The regions of the muscle marked A and B in Figure 1 were used.

generalized pattern of the distribution of the broad fibers in the *pectoralis major* muscle of the pigeon.

For counting the broad as well as the narrow fibers in one and the same region, the same procedure was adopted, except that the image of the section projected on the screen was magnified to about a hundred times, and the sections from the different typical regions of the muscle were used.

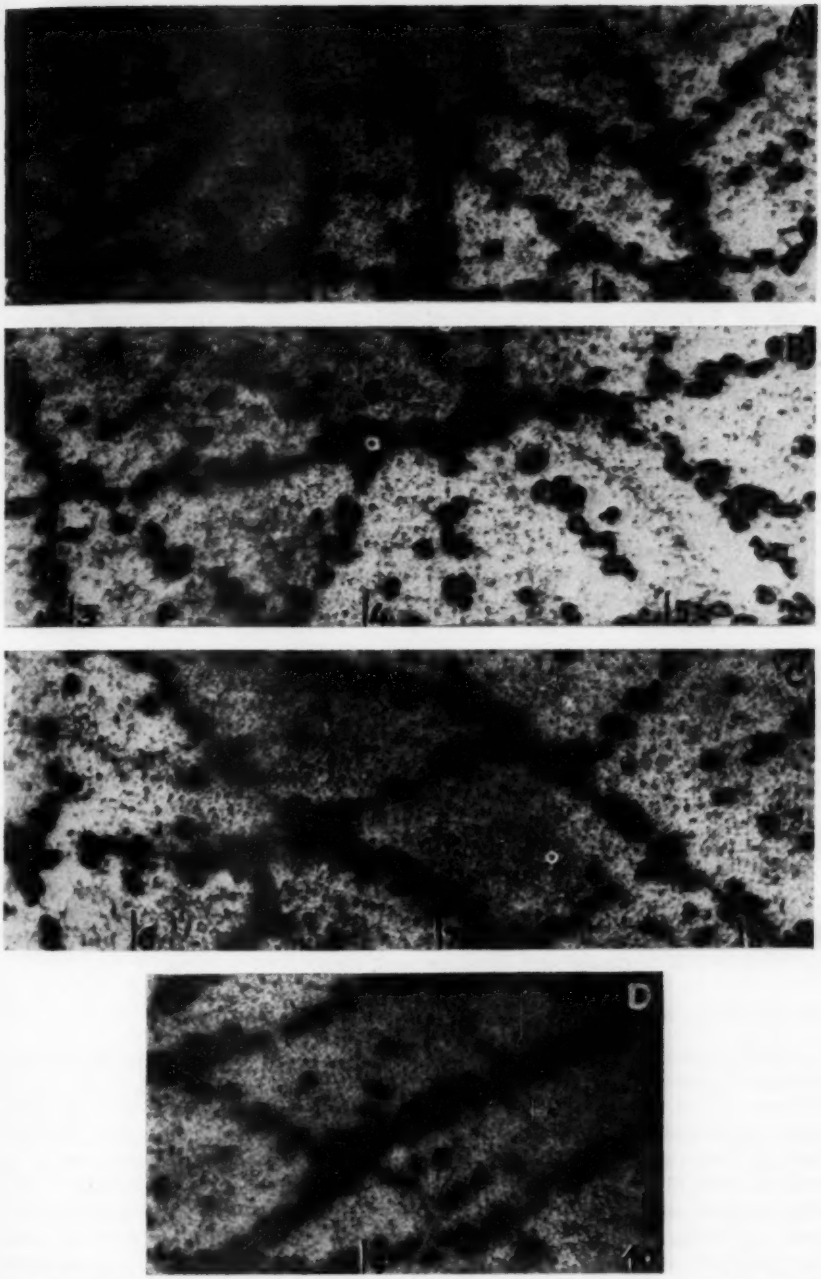

FIGURE 5. Negative prints of the transverse section taken from the region A (Fig. 1) showing the continuous distribution of broad fibers (darker in color) at different depths of the muscle. The numbers 1–10 on microphotographs indicate the depth in mm. from the ventral to the dorsal face of the muscle.

Estimation of fat and glycogen at different depths of the muscle

For the sake of convenience, the region of the muscle (marked A in Fig. 1) on the posteriormost end of the keel was used throughout. In this region the thickness of the muscle is only about 10 mm. and the variation in the distribution of the broad fibers at the different depths of the muscle is gradual. From this region A, a piece about 10 cubic mm. in size was cut out for the estimation of glycogen and a somewhat bigger piece for the estimation of fat. From a region B lateral to A, another piece was cut out and transferred to the freezing chamber of the refrigerator and used later on for studying the distribution of the broad fibers in this region by the method already described.

The muscle piece cut out from region A was mounted on the stage of a freezing microtome so as to obtain horizontal sections. It was frozen hard, the outermost epimysium was peeled off with a pointed forceps or sliced off by a superficial stroke of the microtome knife, and 1-mm. thick slices of the muscle were serially cut. Since all these horizontally cut sections were of uniform and known thickness, each could be said to represent the nature of the muscle tissue at a known depth. The thickness of the sections was not actually measured since the microtome used was a brand new "Sartorius" model and all the possible precautions, such as avoiding the fluctuations in the temperature, were taken so as to obtain sections of uniform and accurate thickness. Each frozen section was immediately transferred to a weighing bottle and dehydrated. The sections to be used for the estimation of glycogen were dehydrated in a vacuum-desiccator at one atmosphere pressure and maintained at 0° C., whereas for fat extraction, sections were dehydrated in an air-oven at 80° C., and finally in vacuum.

The dehydrated sections were weighed and their glycogen content was estimated according to the method of Kemp *et al.* (1954). For the quantity of the muscle used for estimation (about 20–30 mg. per dry weight) it was found necessary to dilute the glycogen extract in the deproteinizing solution to 10 ml. The color developed was measured on the Beckman spectrophotometer (DU model) at 520 μ. For the estimation of fat the dehydrated material was ground and, after weighing, transferred to a fat-extraction thimble. The fat was extracted in the Soxhlet apparatus with 1:1 ethanol-ether mixture (George and Jyoti, 1955). About 70–100 mg. of dehydrated muscle were used for each estimation.

The estimation of glycogen in the two types of fibers

Small pieces from the breast muscle of a decapitated pigeon were cut out and dropped in previously chilled 80% methanol and left undisturbed at − 10° C. for 24 hours. The fibers from the muscle thus preserved were teased out in methanol under a binocular dissection microscope with watch-maker's forceps. The two types of fibers were isolated and transferred to two separate containers containing methanol and fitted with air-tight glass lids and stored in the refrigerator. Sufficient numbers of fibers which would yield about 2–5 mg. in dry weight were isolated and collected for each estimation. These fibers were then removed from the methanol solution, dehydrated in vacuum and weighed on a microbalance. Glycogen was estimated, as already mentioned, by the micromethod of Kemp *et al.* (1954).

Results

Figure 5 presents a typical picture of the distribution of broad fibers in the muscle. In each fasciculus the broad fibers are mainly concentrated towards the periphery. This pattern is maintained throughout the muscle. In regions of the muscle where there are larger numbers of broad fibers or lesser numbers of narrow fibers, the fasciculi have a smaller cross-sectional area with broad fibers closely packed along their borders without any intervening narrow fibers. The number of broad fibers per square mm. in the different regions of the muscle is shown in Figure 2. The relation of the number of broad fibers to that of the narrow ones per square mm. is shown in Figure 3. From both these, the number of broad fibers, as well as the number of narrow fibers per square mm., in any region of the muscle could be approximately determined.

The variation in the metabolite load and the number of broad fibers per square mm. at different depths of the muscle are indicated in Table I, while in Figure 3 the same data are utilized to show the relation between the structure of the muscle

Table I

The number of broad fibers per square mm. and the percentage of fat and glycogen at different depths of the breast muscle of the pigeon. (The portion of the muscle marked A in Fig. 1 was used. The figures indicate the average values of six sets of readings)

Depth of the muscle in mm. (starting from the ventral face)	Number of broad fibers per square mm. ±S.D.	Percentage per dry weight of the muscle ±S.D.	
		Glycogen	Fat
0–2	90 ± 14	3.655 ± 0.275	10.289 ± 1.942
2–4	63 ± 8	3.175 ± 0.054	12.095 ± 1.056
4–6	48 ± 3	3.102 ± 0.127	14.632 ± 1.752
6–8	51 ± 4	3.409 ± 0.184	13.250 ± 0.571
8–10	72 ± 9	3.588 ± 0.236	11.743 ± 0.572

and the metabolite load. The number of narrow fibers for the corresponding number of broad fibers was calculated by using the formula of the regression line in Figure 3 and the ratio of the area occupied by the broad fibers to that of the narrow fibers in square mm. was determined by using the mean value of the diameter of these fibers. The diameter of the broad fibers is 69.00 ± 14.00 μ (1000) and that of the narrow fibers is 30.11 ± 6.56 μ (2000). The figures given in parentheses indicate the number of fibers measured from the fresh frozen sections taken from the various regions of the muscle.

The values of the glycogen content of the broad and narrow fibers, calculated on the dry weight of the muscle preserved in methanol, are, respectively, 10.240 ± 0.093% and 2.464 ± 0.311% (each value is the mean of three readings). Methanol removes much of the fat (mainly from the narrow fibers) and some of the amino acids.

Discussion

It has been known that in many active muscles, the muscle fibers towards the periphery become larger in diameter and lighter in color, compared to those in

the interior. In such muscles even in the individual fasciculus, the light fibers are situated towards the periphery. In the pigeon breast muscle, the white broad fibers and the red narrow fibers show a somewhat similar distribution pattern but these fibers differ from the light and dark fibers of the other muscles in that they are sharply differentiated into two distinct types without any intermediate forms. The broad fibers are glycogen-loaded and poor in fat inclusions and mitochondria, whereas the narrow fibers are fat-loaded and have a high mitochondrial content and are poor in glycogen (George and Naik, 1958a, 1958b).

In a single muscle uneven distribution of metabolites has been long since realized. To reduce such localized variation to the minimum, customarily a large piece of muscle is utilized for the estimation of metabolites. Present work shows that in a muscle like the *pectoralis major* of pigeon having heterogeneous cellular elements, variation in metabolites in the different regions of the same muscle and even in a single fasciculus is quite large. Needless to say, what applies to glycogen and fat might equally apply to other chemical constituents in which the two types of fibers differ.

A general belief that the muscle fibers towards the periphery of the muscle are more active than those in the interior and, due to higher activity, increase in diameter, does not seem to hold good, at least in the case of the *pectoralis* of pigeon. Undoubtedly, the red fibers of pigeon breast muscle, due to their remarkably well developed enzyme systems, play a major role in effecting the sustained contractions of the muscle. In white fibers, on the other hand, the oxidative processes are not developed or developed only to a negligible extent, in that the dehydrogenase activity in these fibers, as shown by histochemical method, is negligible or nil (George and Scaria, 1958b). All the same, the white fibers are not inactive elements of the pigeon breast muscle. In the normal animal they show no signs of atrophy. A glycerinated white fiber of pigeon breast muscle contracts in the same manner as a glycerinated red fiber of the same muscle on the addition of ATP. The study on the reactions of these two types of fibers to experimentally induced disuse atrophy has yielded significant results. When the movement of the humerus is restricted for three months by a plaster cast, the white fibers in the deeper layer of the muscle show acute sign of atrophy whereas the red fibers appear practically unaffected (George and Naik, unpublished data). These findings suggest the possibility of some differences in the mechanical properties of the two types of the fibers and in that case some physical factors may underlie the distribution pattern of the two types of fibers in the muscle.

Denny-Brown (1954), has shown that a single nerve in the breast muscle of pigeon can innervate both, the red as well as the white fibers. Since the activity of these muscle fibers must be conditioned by the fundamentally different chemical system in them, it is difficult to believe that the amount and the mode of activity performed by these two types of fibers are the same. In what exact manner the white fibers contribute to the activity of the muscle is far from clear and as a prelude to such an understanding, an extensive study of these fibers is essential. For such a study Figure 2 can be a useful guide. Moreover, the method used in the present work to study the variation in the metabolite load in relation to the variation in the fiber make-up of the muscle, can be used for studying the distribution of various constituents such as enzymes, amino acids and minerals in the muscle.

We are grateful to the members of the staff and the technicians of the Departments of Chemistry and Statistics, Faculty of Science, Baroda, for their unfailing assistance in completing this work. One of us (R. M. N.) is indebted to the Ministry of Education, Government of India, for the award of a Senior Research Scholarship.

SUMMARY

1. The relative distribution pattern of the red and white muscle fibers in the breast muscle of the pigeon is studied.

2. There exists a direct relation between the distribution of metabolites and that of the two types of fibers in the different regions of the muscle.

3. Quantitative estimation of glycogen in the two types of fibers confirms the higher concentration of glycogen in the white fibers.

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THE EFFECT OF OSMOTIC STRESS ON THE IONIC EXCHANGE OF A SHORE CRAB

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The decapod Crustacea have received considerable attention with regard to their ability to regulate the inorganic ions of their blood (Krogh, 1939; Robertson, 1949, 1953, 1957; Prosser *et al.*, 1950). Prosser *et al.* (1955) studied responses of the shore crab *Pachygrapsus crassipes* to different concentrations of sea water. The chief concern of their study was to determine the changes in the ionic concentrations of blood and urine which were effected by altering the concentration of the external medium from normal. Determinations on the total losses and gains of the respective ions between animal and medium were not made nor were the effects of desiccation on ion concentrations in urine or blood determined. This information would be of special interest in the case of a semi-terrestrial crab such as *Pachygrapsus*.

Gross (1958) demonstrated that when *Pachygrapsus crassipes* was placed under osmotic stress, the principal exchanges of potassium were between the medium and a source of potassium other than the blood, not mainly between blood and external medium. Also, evidence was produced that an extra-vascular pool participates in sodium exchanges between crab and medium. This paper will produce further evidence that extra-vascular salt pools in *Pachygrapsus* contribute to ionic exchanges with the medium, special attention being paid to calcium and magnesium. The effects of desiccation on the ionic concentration of urine and blood in *Pachygrapsus* will be revealed and data confirming the findings of Prosser *et al.* (1955) will be produced.

Material and Methods

The subject of this investigation, *Pachygrapsus crassipes* Randall, was collected at Laguna and Dana Point, California. All specimens were between molts, and were mature, none being smaller than 20 gm.

Urine was sampled by inserting a micropipette into the excretory pores. Blood was obtained by puncturing the cuticle of the leg joints with a micropipette. Sodium and potassium were measured by means of a Beckman flame photometer. Urine and blood were measured and diluted appropriately before being used directly in the flame photometer (Gross, 1958). Samples as small as 0.05 ml. thus could be analyzed to an accuracy of 2% for sodium and 10% for potassium at the minimum concentrations measured in this investigation. Thus, before and after treatment samples of blood from the same crab could be analyzed for sodium and potassium. Calcium and magnesium were determined by titration with ethylenediamine tetra acetic acid (EDTA) a method described by Schwarzenbach *et al.* (1946) and Knight (1951). This method requires about 0.25 ml. of urine and

about 0.50 ml. of blood. Because of the relatively large volume needed, repeated blood samples on the same specimen were not taken. Urine samples were diluted to 100 ml. and titrated directly. Media were titrated directly. Blood samples were dialyzed against distilled water and the dialysate was titrated, a correction being applied for the content of the dialysis bag. This process, which was not necessary in the case of urine, gave a more distinct end-point than titrating the diluted blood directly. Calcium and magnesium thus could be recovered within an accuracy of 5% for the minimum concentrations measured.

TABLE I

Effects of stress on ionic concentrations of blood and urine in Pachygrapsus

	Treatment											
	50% sea water			100% sea water			150% sea water			Desiccation		
	Mean	S.D.	No. crabs	Mean	S.D.	No. crabs	Mean	S.D.	No. crabs	Mean	S.D.	No. crabs
Sodium (mEq./l.)												
Blood	397	24	37	*483	17.3	36	582	34	30	536	27.4	32
Urine	380	60	37	378	64.0	15	353	106	30	297	104	15
U/B ratio	0.96	0.14	37	0.78	0.14	15	0.63	0.16	30	0.56	0.17	15
Medium	232			464			696					
Potassium (mEq./l.)												
Blood	7.36	1.4	37	*7.43	0.72	36	10.23	1.48	30	11.5	1.63	32
Urine	9.95	3.5	37	7.76	1.35	15	9.59	1.13	30	14.8	3.18	15
U/B ratio	1.45	0.50	37	0.82	0.19	15	0.94	0.33	30	1.34	0.50	15
Medium	4.9			9.8			14.7					
Calcium (mEq./l.)												
Blood	34.8	7.9	24	29.6	5.9	44	36.4	4.8	30	45.2	10.7	36
Urine	32.7	7.1	31	36.0	6.3	15	47.9	5.2	20	44.4	7.36	12
U/B ratio	0.98	0.13	23	1.17	0.20	15	1.33	0.18	29	1.07	0.33	12
Medium	10.0			20.0			30.0					
Magnesium (mEq./l.)												
Blood	13.6	5.36	24	20.0	6.1	44	27.1	4.22	30	28.5	15.9	36
Urine	70.5	41.1	31	236	87	15	408	122	29	424	144	12
U/B ratio	5.62	4.52	23	13.6	5.3	15	15.4	4.44	29	23.6	10.9	12
Medium	52.0			104			156					

* Gross (1958).

In order to measure the exchange of ions between animal and external medium, crabs freshly removed from normal sea water were weighed, and blood was sampled for sodium and potassium determinations. The crabs then were rinsed in water of the salinity to which they were to be exposed, then immersed in a small volume (50 ml.) of that same water for a period of about 24 hours. Adequate precautions were taken against water loss by evaporation. Values concerning sodium and potassium exchanges (Table II) have been reported previously (Gross, 1958)

and include some data on animals immersed 24–48 hours in 100 ml. The crabs could raise themselves out of the water and therefore were not completely immersed at all times. After a period of about 24 hours, the animals were removed from the media and their blood and urine sampled for the analysis of sodium, potassium, calcium and magnesium. Likewise the media were analyzed for these ions.

Other crabs freshly removed from normal sea water were weighed, then desiccated for a period of about 48 hours for a loss of about 7% original weight. After this treatment the blood and urine were analyzed for the above four cations.

TABLE II

Relative ion changes in blood and external medium caused by altering external medium from normal

	Mean of ratios*	50% sea water	S.D.	No. crabs	150% sea water	S.D.	No. crabs
Na	$\frac{\text{Blood change (mEq./l.)}}{\text{Medium change (mEq./l.)}}$	2.56	0.82	28	2.63	0.80	25
K	$\frac{\text{Blood change (mEq./l.)}}{\text{Medium change (mEq./l.)}}$	0.56	0.43	20	1.00	0.70	24
Ca	$\frac{\text{Blood change (mEq./l.)}^{**}}{\text{Medium change (mEq./l.)}}$	0.93	3.23	22	0.78	0.66	28
Mg	$\frac{\text{Blood change (mEq./l.)}^{**}}{\text{Medium change (mEq./l.)}}$	0.85	0.89	24	0.64	0.40	26

* Change in medium for all ions is corrected to a volume equal to the weight of the crab.

** Blood change for calcium and magnesium equals the difference between mean of normal crabs and the observed blood concentration after treatment for each crab. Medium change is the observed concentration change in the medium after treatment for each crab.

Analyses of blood potassium and sodium were made before and after desiccation on individual crabs.

RESULTS

Table I presents the urine and blood concentrations of sodium, potassium, calcium, and magnesium after the following treatments: a) immersion in normal sea water; b) immersion in 50% sea water; c) immersion in 150% sea water and d) desiccation for a water loss of about 7% body weight. Comparing the blood values after immersion in 100% sea water with those of Prosser *et al.* (1955), sodium and calcium appear in agreement. However, the potassium (7.43 mEq./l.) and magnesium (20.0 mEq./l.) values are considerably less than those reported by the above workers (12.1 mEq./l. and 58.4 mEq./l., respectively). On the other hand Schlatter (1941) reported blood ion concentrations for this same species which agree closely with the values of the present investigation.

It should be emphasized that the indicated stress media (Table I) represent only the initial sea water concentrations, and that these necessarily were altered by exchanges of salts with the animal. However, an accurate knowledge of the sustained osmotic gradient and the final blood concentrations is of little meaning in this investigation, since as described above, the animals were able to raise them-

selves out of the water. The main objectives of this study are to demonstrate: 1) the degree to which a blood ion change is reflected in the external medium and 2) the role of the antennary glands in controlling the ion content of the animal. It also should be pointed out that in this crab alterations in the blood concentration in aqueous media are effected by salt exchanges, not water (Gross, 1957).

Data in Table I, however, do reveal something of the ability of *Pachygrapsus* to regulate ions in the different sea water concentrations. Thus blood sodium is held above the sodium concentration of the dilute medium and normal sea water, but below the concentration of the hypertonic medium. Blood potassium is held above the concentration of the dilute medium, but below the concentration of normal sea water or the concentrated medium. Gross (1958) reported that when *Pachygrapsus* was immersed in a small volume of 50% sea water, the blood potassium remained less concentrated than the medium potassium. However, these animals were immersed for longer periods than those reported in the present studies (Table I) during which time the animal lost more potassium and the medium gained potassium. Table I also shows that the blood calcium remains more concentrated than the medium calcium for all treatments. Blood magnesium, on the other hand, is less concentrated than the medium magnesium for all aqueous conditions. All four ions increase under conditions of desiccation.

The ratios, urine concentration/blood concentration (U/B ratio), for each respective ion suggest the role of the antennary glands in the ion regulatory mechanism. Values in Table I are means of U/B ratios observed in individual specimens, not ratios of means. Thus all the mean U/B ratios for sodium are less than one, indicating that the antennary glands do not regulate sodium under this set of conditions. That is, sodium is not eliminated effectively when the gradient between blood and medium favors a gain; nor is it conserved effectively when the gradient favors a loss to the medium (mean U/B ratio in 50% sea water = 0.96).

With respect to potassium the mean U/B ratio is less than one when the crab is immersed in 100% or 150% sea water. Thus the antennary gland does not regulate potassium for this set of conditions. In 50% sea water the mean U/B ratio is 1.45 which means, if anything, potassium is being wasted when it is needed. However, for conditions of desiccation the mean U/B ratio is 1.34 which is significantly greater than one, $P < 0.01$. If then there were sufficient production of urine under conditions of desiccation, the antennary glands would tend to keep the blood concentration of potassium at a normal level.

With respect to calcium the mean U/B ratios for crabs immersed in 50% sea water or subjected to desiccation are not significantly different from unity. Thus the antennary glands are ineffective as regulators of calcium for these two conditions. On the other hand, after immersion in 150% sea water the mean U/B ratio is 1.32 which is significantly different from one, $P < 0.01$. In normal sea water the U/B ratio is 1.17, again being significantly greater than one, $P < 0.01$. Thus, the antennary glands might have a small role in regulating calcium, but in no sense as large a role as they have for magnesium.

Data in Table I demonstrate that the mean U/B ratios for magnesium under all conditions studied are much greater than unity. Even after immersion in 50% sea water, the mean ratio is 5.62. However, it should be pointed out that even in this diluted sea water the gradient between blood and external medium favors the

uptake of magnesium. Also, it will be noted that the mean ratio under conditions of desiccation is 23.6 which suggests that the urine concentration depends on the blood concentration, not entirely on the rate of influx from the external medium.

The data presented in Table I concerning the treatments in aqueous media are qualitatively in general agreement with the findings of Prosser *et al.* (1955), particularly with regard to the role of antennary glands in the regulation of magnesium. Quantitatively the data presented in Table I differ somewhat from those reported by Prosser *et al.* (1955). However, precise comparison should not be attempted because of differences in experimental procedure. For example, crabs of the present investigation were immersed directly in small volumes of stress media for a maximum of about 24 hours. The data presented by the above workers were obtained on animals gradually acclimated to osmotic stresses for a period of at least 5 days in relatively large volumes of media.

On the other hand there are certain differences which warrant attention. Normal blood potassium and magnesium differences already have been mentioned above. It will be observed that blood calcium after immersion of the animal in 50% sea water (34.8 mEq./l.) is higher than it is for animals from normal sea water (29.6 mEq./l.). These means are significantly different; $P = 0.01$. Prosser *et al.* (1955) showed decreases in blood calcium in 50% sea water which, of course, would be expected. It was thought that perhaps the increased blood calcium resulting from immersion in dilute sea water was an effect of the small volume of medium. Therefore, blood calcium of crabs immersed in large volumes (about 700 ml.) of 50% sea water for 24 hours was determined. The mean blood calcium of 24 crabs thus treated was 30.9 mEq./l., S.D. = 9.0. This is not significantly different from the mean (34.8) obtained by the other treatment; nor is it signficantly different from the average blood calcium of normal crabs. These workers also called attention to the inverse relationship between the urine sodium concentration and the blood sodium concentration. That is, the urine sodium of animals immersed in concentrated sea water was less concentrated than that of animals immersed in normal sea water, which in turn was less concentrated than that of animals immersed in dilute sea water. The means for urine sodium after treatment in the three aqueous media (Table I) cannot be shown to be significantly different, but the U/B ratios do suggest the same phenomenon. That is, the ratios decrease as the animal is placed in increasing concentrations of sea water. These ratios are all significantly different from each other; $P < 0.01$. The U/B ratio for the desiccated crabs is not significantly different from the U/B ratio in crabs exposed to concentrated media, but is significantly different from the ratios obtained for crabs given the other treatments; $P < 0.01$.

Data in Table II demonstrate the ionic changes that occur in the medium when a given change in the blood is effected. The measurement of calcium exchanges with stress media was complicated by the fact that this ion is lost in significant amounts when the animal is immersed in normal sea water. Such was not the case for the other ions. It became necessary, therefore, to apply a correction to the calcium exchanges, based on an average loss to normal sea water by 30 crabs. This amounted to 0.5 mEq./l. per gram of crab for a 24-hour period in 50 ml. of medium. It was thus necessary to assume that this *normal* loss is constant in all concentrations of sea water, an assumption which subjects the values for calcium change in the medium to considerable error.

The values for sodium and potassium have been reported previously (Gross, 1958) and represent means of the ratios, blood change (mEq./l.)/medium change (mEq./l.), in individual crabs where the blood change is the difference between the concentration before treatment and the concentration after treatment. For calcium and magnesium the values in Table II also represent means of the ratios, blood change (mEq./l.)/medium change (mEq./l.), in individual crabs, but since only one sample of blood could be extracted from single specimens for calcium and magnesium determinations, the blood change (mEq./l.) in the ratio for calcium and magnesium equals the difference between the observed blood concentration after treatment and the average blood concentration for crabs from normal sea water.

With respect to sodium, the mean ratios are greater than 2.5 in both 50% and 150% sea water. The response to hypertonic stress and hypotonic stress seems to be symmetrical. With respect to potassium the ratio is unity or less; while it is 0.56 for crabs immersed in 50% sea water, it is 1.00 for crabs immersed in 150% sea water. However when ion exchanges were measured in crabs transferred from 50% to 150% sea water or vice versa, a symmetrical response for potassium

TABLE III

Ion increase in blood caused by desiccation

	No. crabs	Mean change in concentration (% original) per 1% body weight loss by evaporation	S.D.
Na	84	+2.20	0.71
K	50	+8.68	11.75
Ca	34	+5.47	4.23
Mg	35	+3.87	9.42

exchanges is observed, the mean ratio, change in blood (mEq./l.)/change in medium (mEq./l.), being about unity in both extreme stresses (Gross, 1958).

The mean ratio for calcium and magnesium is less than one for all treatments. Attention should be called to the large variance for the calcium ratio, following immersion in 50% sea water. It also should be mentioned that the ratio, mean blood change (mEq./l.)/mean medium change (mEq./l.), is $\frac{+5.2}{-1.82} = 2.87$, the signs of the numerator and denominator being opposite to expectation. Not only does the average value for the blood calcium increase after treatment in dilute sea water, but the medium apparently loses rather than gains calcium. The difference between the mean of the ratios (0.93) and the ratio of the means (2.87) can be explained on the basis of the large variance.

Table III reveals ionic changes that occur in the blood when *Pachygrapsus* is desiccated for a loss of about 7% body weight. The sodium and potassium values, again, have been reported previously (Gross, 1958) and represent averages of changes in individual crabs, where the blood concentration change was determined by before- and after-treatment readings on the same individual. The values for calcium and magnesium are means of blood concentration changes for individual

crabs, but since only after-treatment blood samples were taken, the blood change for these two ions is represented by the difference between the observed concentration in an animal following desiccation and the mean blood concentration of the respective ions in crabs from normal sea water. In Table III it can be seen that the average change for sodium is less than the values for the other ions. While the potassium and calcium changes are significantly greater than the sodium change, $P < 0.001$, the mean magnesium change cannot be considered significantly different from the sodium change. It will be explained below that blood ions which increase more in concentration than blood sodium probably shift from a salt pool (perhaps the intra-cellular space) into the blood when the animal is desiccated.

Discussion

The ratios, blood change (mEq./l.)/medium change (mEq./l.), presented in Table II suggest that the principal exchanges of potassium, calcium, and magnesium between animal and medium are not ultimately between blood and external medium. A ratio of unity means that the concentration change in an external medium which is equal in volume to the animal is identical to the concentration change in the blood. Of course, much of the animal's volume is isolated from the osmotic and ionic processes which occur in the blood. Thus for a ratio of unity, the actual loss or gain of ions with the medium would be greater than the loss or gain of ions in the blood. Therefore a source other than the blood must be contributing to these exchanges. These ratios also can be expressed as "apparent volume of distribution," using the equation $V = M/P \times 100$ (Gross, 1958) where:

V = "apparent volume of distribution" in % body weight;

$$M = \frac{\text{weight of medium}}{\text{weight of animal}};$$

$$P = \frac{\text{change in blood ion concentration (mEq./l.)}}{\text{change in medium ion concentration (mEq./l.)}}.$$

Thus, the "apparent volume of distribution" for sodium is 38.5% body weight and for potassium, calcium and magnesium more than 100% body weight, which only can be interpreted as an aggregation of these three ions in some sort of pool where they are much more concentrated than they are in the blood. This also means that the extra-vascular pools ultimately contribute more to potassium, calcium and magnesium exchanges with the medium than does the original blood supply (more than twice as much). At least, in the case of potassium, the pool probably lies mainly in the intra-cellular space, because it is well known that intra-cellular potassium concentrations are high. In the crab *Carcinus* the relative muscle concentrations of sodium, potassium, calcium and magnesium are 50, 120, 11 and 32 (mEq./kg. water), respectively (Shaw, 1955). If this were representative of intra-cellular concentrations, it would seem unlikely that the intra-cellular space harbors the pool for magnesium and calcium. Although the nature of the pools is unknown, it becomes apparent that a change of a blood ion concentration can occur without a loss or gain in the medium. Or exchanges between animal and medium can occur without being reflected in the blood. The probable exception to

this is sodium. The "apparent volume of distribution" for sodium was calculated to be 38.5% body weight for the moderate stresses of 50% and 150% sea water. Webb (1940) estimates the blood volume of the crab *Carcinus* as 36% body weight. Thus the calculated volume, 38.5% body weight, which seems close to a reasonable value for blood space, means that the major sodium exchanges are between the blood and external medium. Though there is evidence that a sodium pool contributes to such exchanges when the animal is exposed to extreme osmotic stress, its role is relatively small percentage-wise, compared with the other ions (Gross, 1958). On the other hand sodium contributes about half the ions of the blood; thus the small percentage effect of a sodium pool would nevertheless affect significantly the total osmotic pressure of the blood.

Burger (1957) immersed lobsters in media of abnormally high magnesium concentrations and noted that neither the blood nor the urine magnesium elevated. On this evidence he concluded that the animal was impermeable to magnesium. However, he did not consider the possibility that the magnesium could enter the animal and be fixed outside of the vascular system, a phenomenon which obviously occurs in *Pachygrapsus*.

The variance for the mean of the calcium ratios, blood change/medium change, when the stress was 50% sea water is high. Nevertheless this ratio for calcium (0.93) is significantly less than the mean ratio for sodium (2.56), $P < .025$. It should be emphasized that the mean blood calcium after immersion in 50% sea water was more concentrated than that for crabs from normal sea water. Also, the corrected average change for calcium in the medium indicated a loss rather than the expected gain. Now, it was revealed above that crabs in normal sea water tend to lose calcium, and the average loss in normal sea water was applied as a correction to the medium measurements, assuming that a loss of calcium (probably by way of the gut) would be the same in a stress as in a normal medium, but if there were a curtailment of *normal* calcium output in dilute sea water, then the correction would be too large and falsely could make the sign of the change in the medium negative. It should be mentioned that the observed changes in the medium without correction were all positive. If the sign of the corrected medium change is in error, then the increase in the blood calcium concentration after immersion in 50% sea water could be caused only by contributions from a calcium reservoir.

Data in Table III demonstrate that for a given weight loss by evaporation the average increase in the blood sodium concentration is less percentage-wise than the increase for the other ions. It was concluded by Gross (1958) that such a difference in increase between sodium and potassium under conditions of desiccation could not be explained on the basis of sodium exclusion from the blood. Rather, it was concluded that it represented a shift of potassium ions from extra-vascular spaces into the blood space. Data for calcium presented in Table III suggest that the same phenomenon happens in the case of this ion; values for magnesium are questionable. No adaptive significance can be assigned to such a phenomenon; rather it is interpreted as a physiological failure which imposes a limitation on the terrestrial habits of this crab.

The U/B ratios presented in Table I suggest the role of the antennary gland as an ion regulator. It has been established previously (Prosser *et al.*, 1955)

that this organ is ineffective as an osmotic regulator. Thus, it seems probable that a principal function of the antennary gland is the regulation of magnesium. That is, the U/B ratio with respect to magnesium is much greater than unity. Yet the effectiveness of the antennary glands as magnesium regulators for each experimental condition cannot be known for certain until the volume of urine production is known for each osmotic situation. Thus, even though the urine magnesium is high when the animal is desiccated, it is possible that little or no urine is produced when the animal is removed from an aqueous medium. Nevertheless, the antennary glands may effectively remove magnesium ions from the blood, thus tending to keep the blood levels normal, even though no ions are ejected from the animal.

These studies were aided by a contract between the Office of Naval Research, Department of the Navy and the University of California, NR 104-309.

I wish to thank Mr. David Allison for his able technical assistance. Also I wish to express my gratitude to all those students who assisted in collecting the experimental animals; to Professor Theodore Holmes Bullock for reading the manuscript; to Professor Timothy Prout for his advice concerning the statistical handling of the data and to Dr. Frank Bingham for suggesting the method for the calcium and magnesium determinations.

Summary

1. The effects of osmotic stress on the ion concentration in the blood of the crab, *Pachygrapsus crassipes*, were investigated. Stresses imposed were 50% sea water, 150% sea water and desiccation to a water loss of about 7% body weight.

2. The observed ratios, blood change (mEq./l.)/medium change (mEq./l.), for sodium, potassium, calcium and magnesium after the crab was transferred from normal sea water to 50% or 150% sea water yielded values for "apparent volume of distribution." The average value for sodium was 38.5% body weight, but for the other three ions was at least 100% body weight.

3. The large values for "apparent volume of distribution" in the cases of potassium, calcium and magnesium indicate that these ions are contained in extravascular pools in greater concentrations than they are in the blood and that these pools participate in ion exchanges between animal and medium. Thus, a concentration change can occur in the blood without being reflected in the medium or vice versa.

4. Calcium is lost to the medium by *Pachygrapsus* when it is immersed in normal sea water. Blood calcium increases when a crab is transferred from normal sea water to dilute sea water.

5. When *Pachygrapsus* is desiccated, the blood concentrations of potassium, calcium and magnesium average greater increases than does the sodium concentration. This suggests that potassium, calcium and possibly magnesium shift from an extra-vascular pool into the blood space. The phenomenon is interpreted as a physiological failure and a factor which may limit the terrestrial life of this species.

6. The ratio, urine concentration (mEq./l.)/blood concentration (mEq./l.), for the respective ions suggests the role of the antennary glands as ion regulators

under the various stress conditions. Thus the antennary glands were found to be relatively ineffective as regulators of sodium, potassium and calcium for all conditions studied. The U/B ratio for magnesium averaged 5.62 when the crab was immersed in 50% sea water; 13.6 for normal sea water; 15.4 for 150% sea water and 23.6 when the crab was desiccated. These high ratios suggest that a principal role of the antennary glands is magnesium regulation.

7. The volumes of urine production which have not been measured must be known before the effectiveness of the antennary glands as magnesium regulators can be determined.

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HISTOLOGY AND METABOLISM OF FROZEN INTERTIDAL ANIMALS[1]

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Many invertebrate animals are normally exposed to environmental temperatures far below the freezing point of their body fluids. Although supercooling may sometimes be a factor in survival (Salt, 1950; Ditman *et al.*, 1942; Scholander *et al.*, 1953), freezing occurs in nature among insects (Asahina *et al.*, 1954; Scholander *et al.*, 1953), shore animals (Kanwisher, 1955), and other groups (Luyet and Gehenio, 1940.) On the shore during winter, for example, freezing and thawing occurs twice a day when the animals are exposed to the cold air by the tide. Intertidal animals in the Arctic may be frozen for as long as 6 months (Kanwisher, 1955). The survival of these animals depends on their being able to have most of their body water turned to ice. It is remarkable that no injury is produced in a living system when more than half of its bulk is changed to a crystalline solid. I am reporting here some investigations on the histology and metabolism of these intertidal animals.

Histology

In the freeze-drying histological technique, tissue is cooled very quickly with liquid nitrogen. Freezing occurs so fast that ice crystals do not have time to grow very large and cellular organization is very little disturbed. The water is removed by vacuum while the sample is kept cold. The resulting dehydrated tissue matrix is imbedded, sectioned, and stained in a conventional manner. I have used the method here to capture the situation in tissue from shore animals frozen to relatively mild natural temperatures. Comparison with material from unfrozen animals has shown the distortions caused by the freezing.

Animals were collected from the shore at Woods Hole in January and moved to a $-10°$ cold room without thawing. Sections of tissue about 1 mm. thick were cut with a cold knife, held with cold tweezers, and plunged into a vial of isopentane suspended in a container of liquid nitrogen. The isopentane allows a faster heat transfer because it does not boil and form an insulating gas layer. The hard frozen samples were quickly transferred to the already cold dehydrating chamber and vacuum applied for 24 hours at about $-45°$. The dehydrated tissue was then imbedded in de-gassed paraffin already in the chamber with the vacuum still applied. Photomicrographs of 10-micron sections are shown in Figure 1. The unfrozen controls were tissue taken from identical animals that had thawed at room temperature for an hour.

[1] Contribution Number 1013 from the Woods Hole Oceanographic Institution. This study was aided by a contract between the Office of Naval Research and the Arctic Institute of North America.

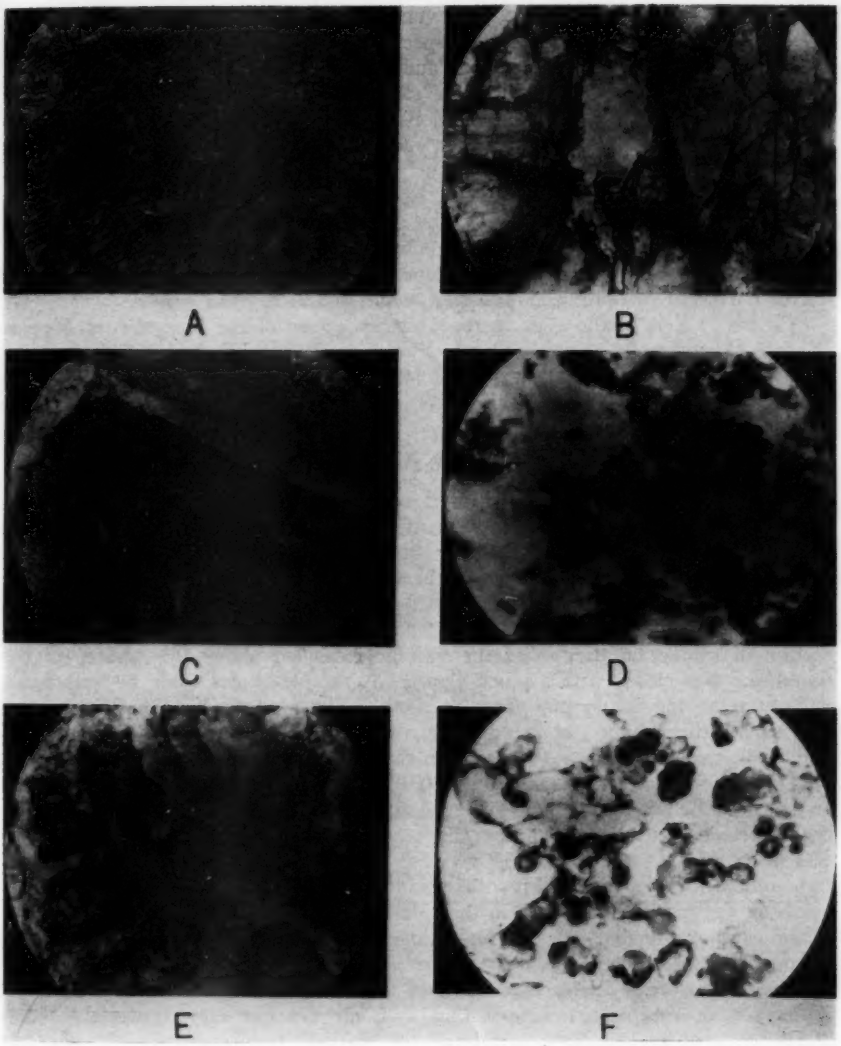

FIGURE 1. Photomicrographs of unfrozen and frozen tissue.

Figure 1, A is the unfrozen foot of the shore snail *Littorina littorea*. The purpose of the randomly arranged muscle fibers is related to the snail's type of locomotion. In the frozen tissue in Figure 1, B, the ice forms in large pockets with a resulting shrinkage and distortion of the cells.

The extreme distortions indicated in the initial results were surprising enough

to warrant the following procedure. The frozen muscle slice was cut in two pieces. One was used as the frozen specimen. The other was warmed for less than a minute on the palm of my hand and then hard frozen in the liquid nitrogen. When sectioned it appeared nearly the same as tissue from an unfrozen animal. Figures 1, A and 1, B are actually sections from this run.

A transverse section of the unfrozen adductor muscle in the oyster, *Crassostrea virginicus,* is shown in Figure 1, C. The parallel muscle fibers are viewed end-on. In its frozen counterpart in Figure 1, D, the fibers are clumped into groups to make room for the intervening ice. The prominent elements that resulted are all about the same size. There may be membranes not visible in the unfrozen muscle to account for this regularity. The same regular clumping was seen in the adductor muscle of two mussels, *Modiolus modiolus* and *Mytilus edulis.*

Figure 1, E is of the eggs in the unfrozen ovary of the blue mussel *Mytilus edulis.* When frozen as in Figure 1, F, the detail is much less distinct but the eggs clearly have shrunken during the formation of the large amounts of intercellular ice. Comparable distortions were seen in other tissues from these and other species.

Metabolism of Frozen Animals

Scholander *et al.* (1953) measured respiration at freezing temperatures by following the decrease in oxygen concentration in a closed volume containing the animal. The same method has been used here. Manometric and volumetric techniques can not be used because of the volume change when water turns to ice.

The snails to be used were frozen in 20-ml. syringes in a cold bath. Only those in which the snail froze while fully extended from its shell were used. A short section of tubing on the tip of the syringe extended above the surface of the liquid and was closed with a pinch clamp. A sample of gas could be withdrawn without removing the syringe from the bath. The plunger was free to move up and replace the volume lost in sampling. Allowance was made for the decreased volume in calculating the rate of oxygen removal.

Duplicate oxygen analyses good to 0.02 per cent were made with the half-cc. analyzer of Scholander (1947). Serial samples were plotted against time and the slope was used in computing the oxygen consumption. The concentration was never allowed to go below 18 per cent in any run. Respiration was assumed to be independent of tension over this small range.

After the snails were placed in the cold bath, at least 6 hours were allowed for phase equilibration between ice and water in the tissues. Previous experience (Kanwisher, 1955) had shown that there was no appreciable increase in ice after this length of time. The syringe was then flushed with cold outside air. A series of oxygen determinations showed that such air did not vary appreciably from 20.94 per cent so this was considered the starting concentration. At intervals ranging from 2 to 120 hours samples were withdrawn with a mercury gas sampler.

Volumetric respirometers (Scholander *et al.*, 1952) were used above 0°. One ml. of sea water was included in the vial with the animals. At 0° such values were in good agreement with those made by gas analysis which is specific for oxygen. The often used and rarely proven hypothesis is thus confirmed that the volume decrease is due to oxygen being consumed.

The respiration temperature data from −10 to +30° C. are plotted in Figure 2. Between 0 and +20°, oxygen consumption shows the usual logarithmic increase

with a Q_{10} of 2 to 3. Above this respiration decreases, probably due to thermal injury. Below 0° the metabolic activity drops sharply with an apparent Q_{10} of about 50.

At −10°, respiration was so low it took 6 days for the snails to consume a measureable amount of oxygen. Even in this length of time the concentration change was smaller than desired for accurate determination. This may account for the greater spread of values at this low temperature. At −15° the empty syringes gave blank values of one-third the oxygen decrease in those containing snails. This may be due to oxidation of grease used on the syringe plunger. It

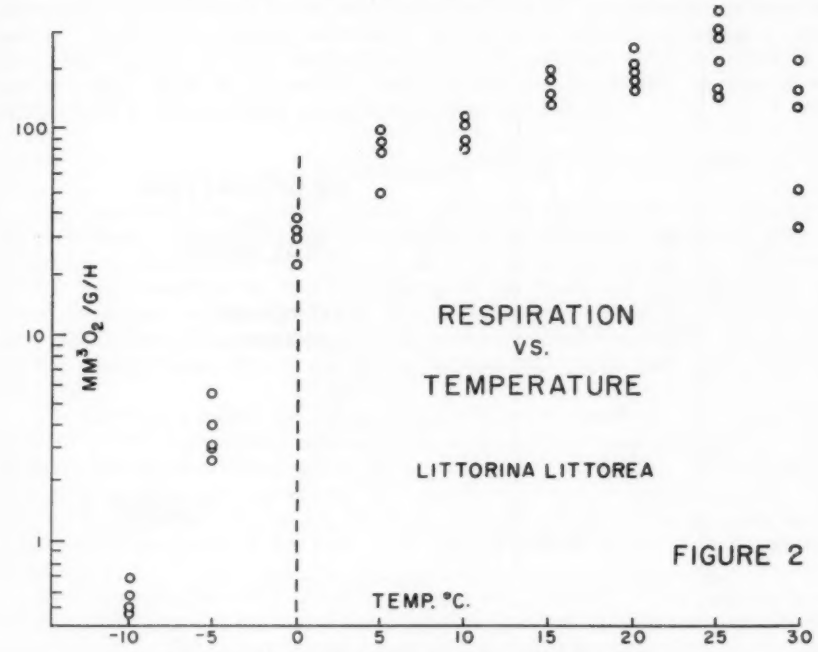

FIGURE 2. Variation of oxygen uptake with temperature.

did not seem that this technique could be trusted on the slower rates to be expected at still lower temperatures.

SALINITY EFFECT ON RESPIRATION

Scholander *et al.* (1953) have given several reasons why the respiratory gas exchange of a frozen animal drops so much more rapidly with temperature than it does above 0° when no ice is present. The ice may act as a diffusion barrier to the gases. The increased viscosity of the body fluids may slow the reaction rates. Finally the increased salinity may directly inhibit the animal's metabolism. No way could be devised to test the first two hypotheses. The respiratory response to increased salinity above 0° can be determined independently of any ice effects.

Higher than normal salinities were made by freezing sea water and using the brine. Dilution with fresh water gave lower than normal salinities. Freshly collected snails were placed in jars containing the different salinities for a minimum of 6 hours before being used. At very high and low values the snails withdrew into their shells. Experience had shown that the operculum blocks respiratory gas exchange so these could not be used.

For the respiration measurements single snails were placed in 20-ml. syringes filled with the desired salinity. The syringes were kept in a constant temperature bath except when sampling. One-ml. samples were removed at convenient intervals and analyzed gasometrically for oxygen by the method of Scholander *et al.* (1955).

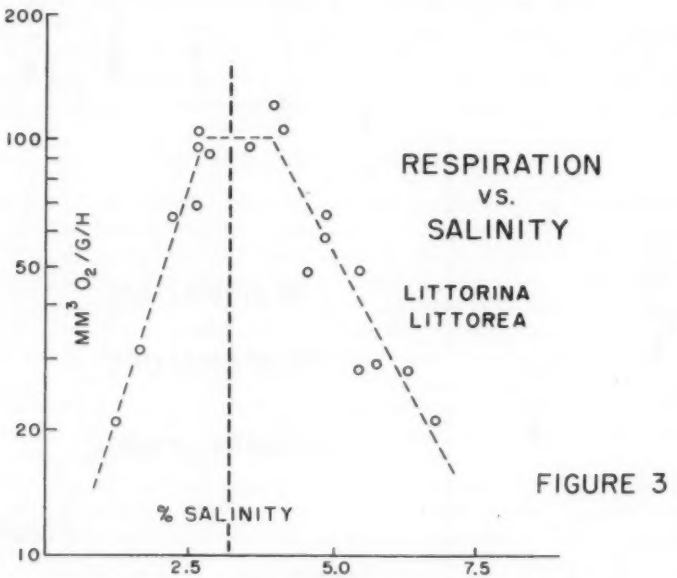

FIGURE 3. Response of oxygen uptake to different salinities.

Since this is a physical extraction of the gases it could be relied on in spite of the water sometimes becoming cloudy with waste products.

As in the low temperature gas analysis method several serial readings were used to indicate the rate of oxygen removal by the snails. Low oxygen tensions were avoided by working in the range of 2.5 to 6 mm.³ of oxygen per ml. The curves showed that respiration was independent of tension over this range.

The variations of oxygen consumption with changes in the external salinity are shown in Figure 3. High salinity depresses the respiration of *Littorina littorea*. This is a reversible effect since the rate increases again when the snail is returned to normal salinity. When the snails withdrew into their shells at higher salinities than shown, no oxygen consumption could be detected. They are apparently able to subsist for long periods anaerobically.

Since freezing occurs throughout the animal, the remaining body fluids in all parts of the animal are concentrated. If any effect of external salinity above 0° is to be related to freezing, it must be shown that the animal is not osmotically regulating. Increased salinity could conceivably be effective in only altering the absorption of oxygen at the surface. Tissues of snails from water of different salinities were analyzed for chlorides. By carefully cracking the shell, the animal could be removed whole. Excess water was mopped off and the weight quickly taken. The water was removed by drying for several hours in a 100° oven. The dry weight then gave the total water by difference. The dry tissue was then digested and titrated for the amount of total chlorides present. This amount was considered dispersed in all the animal water in order to calculate the concentration in the live animal. From the resulting normalities at different external salinities it was clear that internal chloride concentration was proportional to that outside the animal. Any result of externally varied salinity can reasonably be viewed as arising from a corresponding change throughout the animal.

Discussion

Chambers and Hale (1932) observed plant and animal cells freezing under the microscope. They found that ice formation inside the cellular membrane always resulted in the death of the cell. The detailed studies of Asahina and his colleagues (1954) have described the freezing process in the blood and isolated organs of insect pre-pupae and in the intact insects themselves. They also found that intra-cellular freezing is lethal to the cell or tissue. Such results are responsible for the general belief that all freezing in animals and plants takes place outside the cells.

The impressive liquid air freezing experiments of nematodes by Luyet and Gehenio (1940) have almost certainly been an instance of ice within cells. Lack of injury here has been attributed to the very rapid rate of freezing. This vitrification does not allow time enough for ice crystals to grow to a size where they can damage protoplasmic structures. It forms the basis of the histology used here. This phenomenon probably has little to do with the normal ecology of these animals in nature.

Shore animals that are exposed to freezing are in shells. This impedes heat transfer and gives ice crystals time to grow. One can readily see them in an opened animal. The tissues of such an animal are similar in texture and appearance to a frozen piece of meat. It is not surprising when one considers that water makes up three-fourths of the bulk of the animal and four-fifths or more of it may be ice. The photomicrographs presented here show the large amount of distortion necessary at the cellular scale to make room for this ice. Yet this can change back to a more or less normal appearing tissue in 30 seconds as shown in Figures 1, A and B. Siminovitch and Briggs (1949) have related frost hardiness in plants to the ability of water to migrate rapidly in and out of the cells. Unfortunately no equivalent test could be devised to apply this to shore animals.

Littorina littorea was found to remain out of its shell and behave normally in salinities of 2 to 7 per cent. It sharply decreased its metabolism in response to a salinity increase. In Figure 3, the data indicate that doubling the salinity above the optimum decreases the oxygen demand to about a third. As the salinity of

the body fluids is increased by the freezing out of water, oxygen uptake must drop in the same fashion. From the freezing curves in a previous paper (Kanwisher, 1955) 70 to 80 per cent of the water in this species is frozen at −10°. This would result in a Q_{10} from the salinity of about 10. Above 0° the Q_{10} due to the usual temperature effect on reaction rates is between 2 and 3. Combining these one would expect a Q_{10} in the range of 20 to 30 below 0°. The actually observed one is closer to 50. The effect of ice as a gaseous diffusion barrier and that from the loss of water itself may account for the difference. It is felt that the present data do not warrant a more vigorous interpretation. Similar work with intertidal algae (Kanwisher, 1957) has shown that the drying effect of freezing was chiefly responsible for a similar large decrease in respiration. A three-times increase in salinity had little effect on the oxygen uptake of these plants.

Freezing in shore animals to the extent shown here is a normal occurrence twice daily in the winter with no obvious injury to the animal. This freezing hardiness is probably connected with the ability to stand the internal distortions and high salinities that result. The greatly lowered metabolism may be of adaptive significance in severe locations where shore animals are frozen into the ice for months at a time. As such it could represent a considerable saving in food reserves.

Summary

1. Histology of frozen shore animals has shown large pockets of intercellular ice with consequent shrinkage and distortion of the surrounding cells.

2. The Q_{10} drops precipitously in the region of ice formation and may be as high as 50.

3. High tissue salinity without freezing decreases oxygen uptake. Thus the salinity increase that results from freezing is responsible for a large part of the high Q_{10}.

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CHROMATOGRAPHIC ANALYSES OF AMINO ACIDS IN THE DEVELOPING SLIME MOLD, DICTYOSTELIUM DISCOIDEUM RAPER [1]

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The slime mold, *Dictyostelium discoideum* Raper, is a relatively simple biological system in which to study the processes of differentiation and morphogenesis. From a seemingly homogeneous mass of cells (the aggregation mass), there are eventually formed in the mature sorocarp two basic cell types—the stalk cell and the spore cell. The developmental cycle of *D. discoideum* has been described in detail by Bonner (1944) and Raper (1935, 1940) and will not be repeated here.

In the recent literature, studies have been reported that suggest correlations between nitrogen metabolism and the processes of differentiation and morphogenesis in this slime mold. Gregg, Hackney and Krivanek (1954) detected the evolution of ammonia and described changes in several nitrogenous fractions during the life cycle of this organism. In this same study, they suggested that the cellulose of the mature sorocarp was synthesized at the expense of a protein precursor and pointed out that the major nitrogen changes took place while the spore and stalk cells were being formed, *i.e.*, during the culmination process. In addition, Krivanek and Krivanek (1958), using the histochemical technique devised by Francis (1953), demonstrated the occurrence of amine oxidase activity in various regions of the slime mold undergoing differentiative changes. The simultaneous occurrence of changes in nitrogen metabolism and of differentiative and morphogenetic phenomena prompted the present study.

Materials and Methods

The method as outlined by Block, Durrum and Zweig (1955) was used for ascending paper chromatographic determinations of amino acids in the slime mold. Chromatograms, using hydrolyzed and unhydrolyzed tissues, were made of four representative stages of development—migrating pseudoplasmodium, pre-culmination, culmination, and mature sorocarp. In the case of hydrolyzed tissue, individuals in the desired stage of development were isolated and homogenized in 6 N HCl, hydrolyzed for 18 hours, and evaporated over a boiling water bath. The residue was placed in a soda lime desiccator for 48 hours and then taken up in 2 cc. of warm glass-distilled water and filtered. After evaporating the water filtrate, the residue therefrom was taken up in 1 cc. of iso-propanol, the vehicle used in the application of the spot. In the case of the unhydrolyzed tissue,

[1] This research was supported in part by Research Grant E 1453 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

homogenates were made with water and evaporated. The residue was taken up in 1 cc. of iso-propanol and applied to the paper.

The microhomogenizer described by Gregg, Hackney and Krivanek (1954) was used for the preparation of the tissue homogenates. All homogenization took place at room temperature (22° C.). Depending upon the stage of development to be analyzed, the homogenization procedure lasted from thirty minutes to an hour. All evaporation took place over a boiling water bath with the evaporation lasting

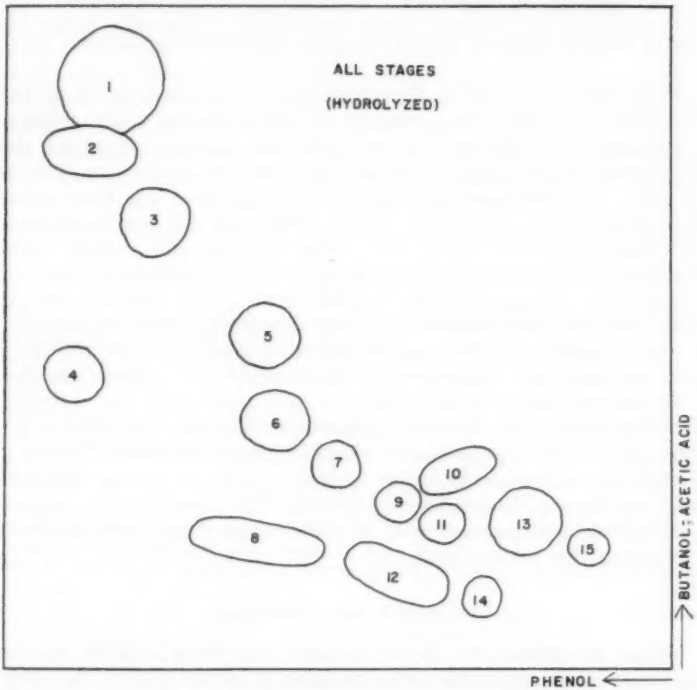

FIGURE 1. Diagram of the results of two-dimensional chromatography on hydrolyzed tissue of *D. discoideum*. Spots are identified as the leucines (1), phenylalanine (2), methionine (3), proline (4), tyrosine (5), alanine (6), threonine (7), histidine (8), glycine (9), glutamic acid (10), serine (11), asparagine (12), unknown (13), cystine (14), and aspartic acid (15).

no more than five minutes in any case. Rupture of virtually all cells was insured by means of periodic microscopic examination of the homogenate.

For both types of analyses, *i.e.*, hydrolyzed and unhydrolyzed, two-dimensional chromatograms were made on Whatman No. 1 filter paper. For the first dimension, n-butanol, acetic acid and water (250, 60, 250 v/v/v) were used as the solvent mixture. For the second dimension, an 80% solution of phenol in water was used as solvent. Development of the spots was accomplished by means of spraying the chromatograms with a solution of 0.3% ninhydrin in 95% ethanol. After

spraying, the chromatograms were allowed to dry in complete darkness for 18 hours. No less than 6 and no more than 10 runs were made for each analysis. In the majority of cases, consistent spot patterns were achieved and only 6 runs were made. However, in those few cases where slight inconsistencies in the patterns were evident, additional runs were made to achieve reproducibility.

Identification of the spots was achieved in two ways. Firstly, R_f values were calculated and compared with the R_f values of known amino acids. Secondly, one-dimensional as well as two-dimensional "control" runs were made using solutions of known amino acids, both singly and grouped, and the loci of spots were compared between the control and experimental series.

Results

Hydrolyzed tissue. Results of the chromatographic studies of amino acids in hydrolyzed tissues of *D. discoideum* are shown in Figure 1. With the exception

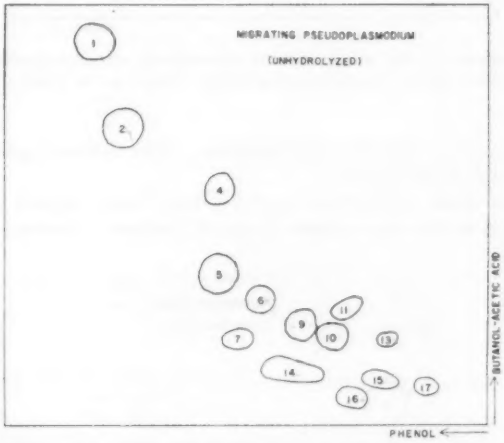

FIGURE 2. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the migrating pseudoplasmodium stage. Identified spots are the leucines (1), methionine (2), tyrosine (4), alanine (5), threonine (6), glycine (9), serine (10), glutamic acid (11), aspartic acid (13), and cystine (15). Spots 7, 14, 16, and 17 are unknowns.

of one spot (no. 13), all spots were identified. The identified spots included the leucines (1), phenylalanine (2), methionine (3), proline (4), tyrosine (5), alanine (6), threonine (7), histidine (8), glycine (9), glutamic acid (10), serine (11), asparagine (12), cystine (14), and aspartic acid (15).

The same spot pattern persisted throughout the four analyzed stages of development. Although no quantitative determinations of the amino acids were made, comparisons of the relative spot intensities afforded some degree of quantification. Glutamic acid presented the most intense color in each stage. Also quite intense, but not to the degree of glutamic acid, were the spots of the leucines, methionine, alanine, threonine, serine, and asparagine. Medium light spots resulted from

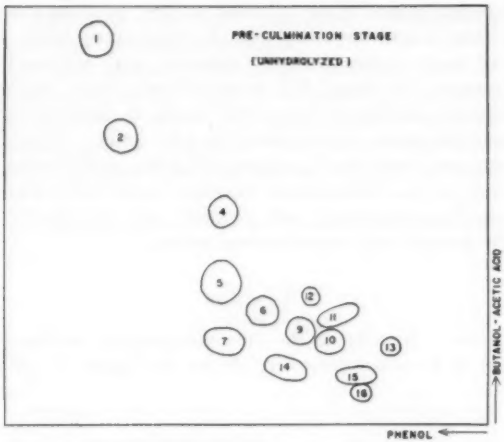

FIGURE 3. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the pre-culmination stage. Spots as in Figure 2, plus spot 12, an unknown.

phenylalanine, tyrosine, glycine, and histidine. The faintest spots were those of proline, cystine and aspartic acid.

In addition to these well-formed spots, a very faint, vaguely-defined spot was occasionally found in the approximate locus of cysteine. Because of its vagueness

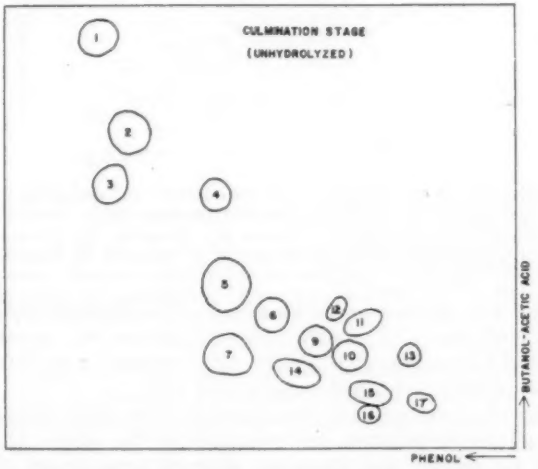

FIGURE 4. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the culmination stage. Spots as in Figure 2, plus spots 3 and 12, unknowns.

and the failure of our controls to show a clear cysteine spot, we cannot state positively either the presence or absence of cysteine.

Unhydrolyzed tissue. Results of the chromatographic studies of amino acids in unhydrolyzed tissue of *D. discoideum* are shown in Figures 2, 3, 4 and 5. Whereas a consistent spot pattern occurred throughout the developmental cycle in the case of hydrolyzed tissue, considerable variability in the spot patterns occurred between the several stages in the case of unhydrolyzed tissue. A total of 17 spots appeared in all or nearly all of the stages of development. However, only ten were identified. They were the spots of the leucines (1), methionine (2), tyrosine (4), alanine (5), threonine (6), glycine (9), serine (10), glutamic acid (11), aspartic acid (13), and cystine (15). The remaining seven spots—3, 7, 8, 12, 14, 16, and 17—were not identified. Presumably these ninhydrin-positive

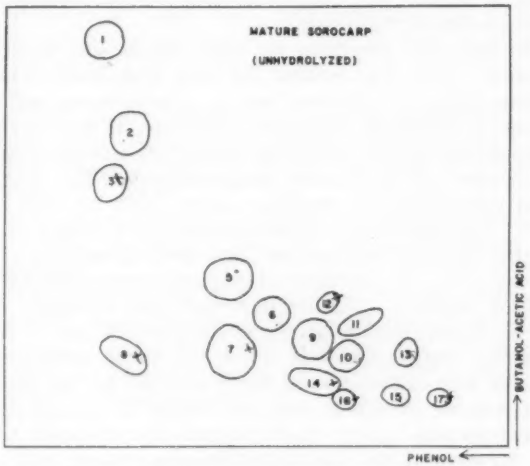

FIGURE 5. Diagram of the results of two-dimensional chromatography on unhydrolyzed tissue of *D. discoideum* in the mature sorocarp stage. Spots as in Figure 2, plus spots 3, 8, and 12, unknowns.

spots were simple peptides. It is possible that these spots were the products of partial hydrolysis by enzymes derived from the cells. However, in view of the rapidity with which the tissues were prepared, this would seem unlikely. Those spots which were evident in all stages of development were 1, 2, 5, 6, 7, 9, 10, 11, 13, 14, 15, and 16. Although spot 12, an unknown, did not appear in the migrating pseudoplasmodium, it did appear in the succeeding three stages of development. Spot 3, also unknown, appeared only in the culmination and mature sorocarp stages, while spot 8, a third unknown, appeared only in the mature sorocarp stage. Spot 4, identified as tyrosine, was present in all stages except the mature sorocarp, and spot 17 appeared erratically—being present in all but the pre-culmination stage. As in the case of hydrolyzed tissue, cysteine could not be definitely ascertained as either being present or absent.

Discussion

In their quantitative studies of the nitrogen metabolism in the slime mold, *D. discoideum*, Gregg, Hackney and Krivanek (1954) demonstrated a decrease in the total nitrogen/dry weight during the transition from the migrating pseudoplasmodium to the mature sorocarp. They attributed this decrease to a decrease in the total extractable protein nitrogen and total unextractable nitrogen components of the slime mold. In addition, they found that ammonia was being given off by the slime mold during its life cycle. In a subsequent study, Gregg and Bronsweig (1956) found a steady increase in the total amount of reducing substances (presumably carbohydrates) as the life cycle progressed. On the basis of these data, it was suggested that the protein fraction of the slime mold served as a precursor for the carbohydrate of the mature sorocarp. However, no indication was made of the possible pathway(s) involved in this conversion. The present study may be suggestive in this respect.

Glutamic acid invariably presented the most intense spot of any of the determined amino acids. This was evident in both hydrolyzed and unhydrolyzed tissues. The deamination of glutamic acid to α-ketoglutaric acid with the corresponding release of ammonia is known. Because of the reversibility of this reaction, it is considered to be one of the prime mechanisms responsible for the interconversion of ammonia and α-amino group nitrogen. The reaction is catalyzed by glutamic acid dehydrogenase, requiring either DPN or TPN as a coenzyme (Meister, 1957). The importance of this reaction, as it relates to *D. discoideum*, lies in the fact that not only has glutamic acid been detected to a high degree in the slime mold, but, also, the liberation of ammonia during the life cycle suggests such a deamination reaction. Further, Krivanek and Krivanek (1958) demonstrated non-specific dehydrogenase activity in the pre-stalk area of the pre-culmination and culmination stages—stages in which the future sorophore sheath (consisting primarily of polysaccharides) is being secreted by the stalk cells as they move apically to become eventually enclosed within the sorophore sheath. This non-specific dehydrogenase activity could logically be attributed to glutamic acid dehydrogenase. By virtue of the relationship between glutamic acid, α-ketoglutarate, areas of dehydrogenase activity, and sites of carbohydrate secretion, there thus can be postulated this link between carbohydrate metabolism and protein metabolism in the slime mold.

The glutamic acid-ketoglutarate relationship, if actually operative in the slime mold, need not be the only link between carbohydrate metabolism and protein metabolism. Aspartic acid, also demonstrated in hydrolyzed and unhydrolyzed tissues of the slime mold, can be deaminated to fumarate, another intermediate in the citric acid cycle (Meister, 1957), thus creating a second possible link between the two types of metabolism. Further, there is the possibility that alanine can undergo deamination forming the Krebs cycle intermediate—pyruvate—as has been suggested by Meister (1957), and serine, as well as cysteine, can undergo the same process yielding ammonia and pyruvate.

The suggested relationships already discussed do not preclude the possibility of other mechanisms relating carbohydrate metabolism to protein metabolism, such as decarboxylation and transamination. There is as yet, however, no evidence to indicate the presence of these mechanisms in the slime mold.

Several of the amino acids of the hydrolyzed tissues appear as well in unhydrolyzed tissue. Consequently, it is not possible to determine whether these amino acids occur as free amino acids only, or also as bound amino acids. However, four amino acids appear only in the hydrolyzed tissue (phenylalanine, proline, histidine, and asparagine). They are considered therefore to exist only in the bound form. The significance of these amino acids with respect to the differentiative process in *Dictyostelium* is at present not apparent.

Studies have recently been initiated to test the validity of the above postulates. These correlative studies will embrace the use of the analogs of the amino acids shown to be present in *D. discoideum*.

Summary

1. The amino acids in hydrolyzed and unhydrolyzed tissue of the slime mold, *Dictyostelium discoideum* Raper, have been determined by means of two-dimensional ascending paper chromatography. Analyses were made on four stages of development—migrating pseudoplasmodium, pre-culmination, culmination, and mature sorocarp.

2. Unhydrolyzed tissue contained the leucines, methionine, tyrosine, alanine, threonine, glycine, serine, glutamic acid, aspartic acid, cystine, and seven unidentified spots, presumably simple peptides. Not all these spots were present in all tested stages.

3. Hydrolyzed tissue contained in addition to the amino acids identified above, phenylalanine, proline, histidine, asparagine, and one unknown spot. All tested stages were identical.

4. The postulate is presented that glutamic acid (and possibly also to a lesser degree aspartic acid, alanine, serine, and cysteine) through deamination may enter the Krebs cycle and form a link between protein and carbohydrate metabolism, the change in balance between protein and carbohydrate being one of the most prominent features of differentiation in this organism.

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SOME ASPECTS OF OSMOREGULATION IN TWO SPECIES OF SPHAEROMID ISOPOD CRUSTACEA

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The internal effects of osmoregulation were studied in two euryhaline species of isopod crustaceans, *Gnorimophaeroma oregonensis* (Dana) and *Sphaeroma pentodon* Richardson. Although a large literature exists on the subject of osmoregulation in Crustacea, only a little of it concerns isopods. Therefore, the present study was undertaken to gain more information in this relatively unexplored area.

Bogucki (1932) studied the ionic composition of the body fluid in *Mesidotea entomon*, which according to Ekman (1953) is an inhabitant of the Baltic and Arctic Seas and several fresh-water lakes in the land area of the northwest Pacific, Siberia, and northern Europe. Bogucki found the body fluid concentration to be hypertonic to the medium in lower salinities, becoming isotonic as the medium approached sea water. Lockwood and Croghan (1957), studying the brackish- and fresh-water races of the same species, found the osmotic behavior to be similar in both races, except that the brackish-water animals could not survive in fresh water. They concluded that the fresh-water race has developed a more effective osmoregulatory mechanism that enables it to maintain the high haemolymph concentrations of the brackish-water race in fresh water. Bateman (1933) found that *Ligia oceanica* maintained its body fluid hypertonic to a medium of about 80 per cent sea water, but swelled and died in 50 per cent sea water. However, Parry (1953), working with *Ligia exotica*, found that in well-aerated sea water, specimens of the species could survive 17 to 30 days in salinities ranging from 50 to 125 per cent sea water. In very concentrated media ($\Delta = 3.46°$ C.), the body fluid was maintained hypotonic to the medium. Menzies (1954), in addition to splitting *Gnorimosphaeroma oregonensis* into two subspecies, *lutea* and *oregonensis*, performed preliminary experiments to test the ability of the two subspecies to survive in various salinities. Specimens of *G. o. oregonensis* taken from 25 per cent sea water and placed in tap water were all dead after one day. Specimens of *G. o. lutea* taken from 1.6 per cent sea water and placed in tap water died slowly over a period of three days. Menzies concluded that *G. o. oregonensis* is probably restricted to sea water, but he was puzzled as to why *G. o. lutea* could survive in sea water, but not in tap water (salts equivalent to 0.3% sea water), which was not very much less saline than the normal habitat water (1.6% sea water). He postulated that *G. o. lutea* required a slight concentration of salts, greater than the tap water used, or that there were toxins present in that medium.

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Materials and Methods

Experimental animals

Gnorimosphaeroma oregonensis is widely distributed over the west coast of North America from Alaska to central California (Menzies, 1954). It also occurs in Hawaii (M. A. Miller, unpublished report). It may be collected intertidally in bays, in estuarine conditions and occasionally populations of the species are found in fresh-water creeks and ponds. Because of its ability to inhabit such a wide ecological range, it was considered to be a suitable experimental type for the further elucidation of osmoregulatory adaptations which enable a marine animal to live in brackish and fresh water.

The following designations will be used for the three habitat groups of *Gnorimosphaeroma oregonensis*. Animals taken from fresh water will be called *G. oregonensis* (FW); those taken from estuarine populations will be called *G. oregonensis* (EF = estuarine form), and those animals taken from intertidal bay populations will be designated *G. oregonensis* (BF = bay form). *G. oregonensis* (FW and EF) equal the subspecies *G. o. lutea* of Menzies. *G. oregonensis* (BF) equals the subspecies *G. o. oregonensis* of Menzies.

Sphaeroma pentodon is known only from San Francisco Bay (Richardson, 1905), Tomales Bay (new locality), and Bolinas Lagoon (new locality), California. It lives intertidally in burrows, which it constructs in mud, wooden logs and pilings, and sandstone. The salinity of the habitats from which it was collected ranged from about 11 per cent sea water to about 96 per cent sea water. *S. pentodon* was included in the study because it is related to *Gnorimosphaeroma oregonensis*, and its range overlaps that of the latter species in parts of its distribution.

Methods

Four principal types of studies were made: (1) Changes in the total osmotic pressure of the body fluid after three, 24, and 48 hours exposure to the experimental salinities were made in order to determine the relative degree and pattern of osmoregulation exhibited by the animals. (2) The animals were weighed before and after exposure to the experimental salinities for 24 hours in order to detect possible changes in weight indicating water gain or loss. (3) Survival tests were run to determine the length of time the experimental animals could live in the experimental salinities. (4) Field checks were made by measuring changes in the body fluid of all but *Gnorimosphaeroma oregonensis* (FW) during a portion of a tidal cycle.

The laboratory experiments were conducted at 16° C., a temperature to which all forms were accustomed. The animals were placed in 60 per cent sea water for 24 hours prior to the start of the experiments. The 60 per cent sea water permitted a common starting salinity for all experimental series, facilitating comparisons.

Experimental salinities

The experimental salinities used in this study were 125, 100, 75, 50, and 25 per cent sea water, and fresh water (salts equivalent to 0.25% sea water by chloride determination). The 100 per cent sea water (salinity = 34.44‰) was collected off

the Marin County coast away from the influence of fresh-water streams. The 25, 50, and 75 per cent sea water solutions were made by diluting normal sea water with distilled water. The 125 per cent sea water solution was prepared by boiling normal sea water, taking care not to precipitate salts. The pH was checked before and after boiling to ascertain that any loss of carbon dioxide was regained by exposure to air. The fresh water was soft creek water collected at Pilarcitos Creek, San Mateo County, California.

Salinity determinations

Salinity determinations on sea water concentrations greater than 25 per cent sea water were made by a short method described by Welsh and Smith (1953). The salinity of sea water diluted to less than 25 per cent normal sea water and fresh water was determined by the standard silver nitrate titration method using the Knudsen Tables (1901).

Melting point determinations

A method devised by Gross (1954) was used for determining the melting point of body fluids. From repeated runs on standard samples, it was found that the concentration of the body fluids could be obtained within an error of about two per cent sea water (0.04° C.).

Body fluid samples (*ca.* 1–2 mm^3.) were collected into prepared melting point capillaries (*ca.* 1 mm. ID × 3 cm. length) which were previously marked with a coded series of dots corresponding to the experimental salinities to which the animals had been exposed. Collection of the body fluid was facilitated by the use of a hand control. After collection, both ends of the capillary were sealed with petroleum jelly and the sample quick-frozen on dry ice.

Survival tests

The ability of the experimental animals to survive for extended periods of time in the experimental salinities was tested as follows: Seventy animals of each experimental group were placed, ten each, in six jars containing the experimental salinities, and one jar containing filtered habitat water. The jars were checked daily for 21 days, and the number of survivors recorded.

Field tests

Changes in the body fluid concentration of *Gnorimosphaeroma oregonensis* (EF), *G. oregonensis* (BF), and *Sphaeroma pentodon* during a 7½-hour period from low to high tide in the field were measured as follows: In the case of *G. oregonensis* (EF), which remains immersed in water during low tide, five body fluid samples and one sea water sample were taken at 1¼-hour intervals. In the case of *G. oregonensis* (BF) and *S. pentodon*, which remain out of the water during low tide, five body fluid samples and five samples of water around the pleopods were collected. The body fluid and pleopod water samples were frozen on dry ice and returned to Davis for determination.

Results

The term "gradient" will be used in the following pages to indicate the difference in concentration (expressed in percentage sea water) between the body fluid and the medium.

Melting point determination of body fluid concentrations

The results of melting point determination of body fluid concentrations are shown in Figure 1. In general, changes in the body fluid concentrations seemed

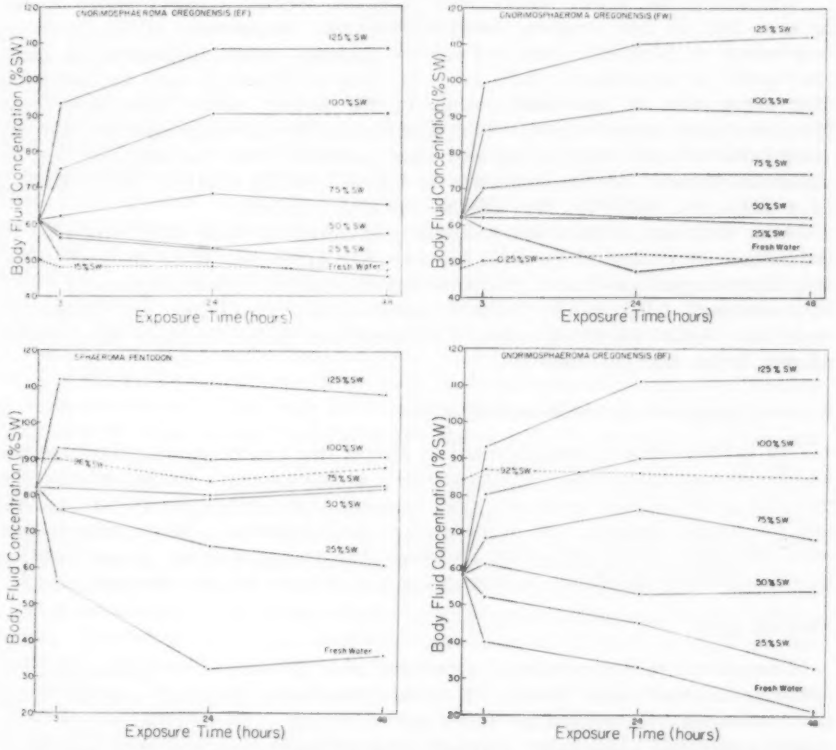

FIGURE 1. Body fluid concentration changes with time in the experimental salinities. The dotted line represents the body fluid concentration changes of animals kept in habitat salinities (controls) indicated.

to be rapid—the major changes occurred within the first three hours of exposure to the experimental salinities.

After 48 hours' exposure, the fresh-water and estuarine forms of *Gnorimosphaeroma oregonensis* maintained their body fluids hypertonic to the medium in 50 per

cent sea water and less, and hypotonic in 75 per cent sea water and above. However, in 75 per cent sea water after 24 hours' exposure, the body fluid concentration values of *G. oregonensis* (FW) were quite variable, ranging between hypotonicity and hypertonicity. Possibly that salinity is close to the medium concentration where the "switch" from hyper- to hypo-osmotic regulation occurs. *G. oregonensis* (BF) maintained its body fluid hypotonic to the medium in 75 per cent sea water and above, and hypertonic to 50 and 25 per cent sea water. Apparently, there was no maintenance of the body fluid concentration in fresh water. In that medium, the body fluid concentration steadily dropped, and after 48 hours, all of the animals were dead.

Comparing the above results with those of Menzies (1954) above it can be seen that in both studies, *Gnorimosphaeroma oregonensis* (BF) (= *G. o. oregonensis* of Menzies) could not survive in fresh water. However, in Menzies' study, *G. oregonensis* (EF) (= *G. o. lutea* of Menzies) were not surviving after three days in tap water, while in the present study, that form lived for several days in fresh water. It is possible that the tap water used by Menzies (unchlorinated well water) contained some unknown toxic substance or had an imbalance of ions. Its ion analysis is as follows: HCO_3, 0.241‰; SO_4, 0.037‰; Cl, 0.029‰; Ca, 0.011‰; Mg, 0.020‰; and Na, 0.078‰.

After 48 hours, *Sphaeroma pentodon* maintained its body fluid hypotonic to the medium in 100 and 125 per cent sea water and hypertonic in the lower salinities. It is interesting to note that *S. pentodon* and *Gnorimosphaeroma oregonensis* (BF) have extremely wide viability limits in terms of the concentration and dilution of their body fluids—surviving within a concentration range (of their body fluids) of over 70 per cent sea water!

Weight changes in the experimental media

No weight changes were detected in any of the experimental animal groups, except *Gnorimosphaeroma oregonensis* (BF) in fresh water. In that salinity, the majority of the animals were very close to death at the end of the 24-hour period, and the weight changes were considered to be subnormal. Those animals that were still active at the end of the 24 hours did not lose weight. It was possible to weigh the animals within an average error of one per cent of their body weight.

Survival tests

The survival experiment was terminated after 21 days. At termination, the estuarine and fresh-water forms of *Gnorimosphaeroma oregonensis* were surviving in all salinities. *G. oregonensis* (BF) was surviving in all salinities except fresh water, where the LD_{50} value (average survival time) was less than two days. *Sphaeroma pentodon* was surviving in all experimental salinities, except fresh water, where the LD_{50} value was 11 days. No unusual mortality was noted among the controls.

Field tests

The results of the field test of body fluid concentration changes during a tidal cycle showed that no significant changes in concentration of the body fluid or water

surrounding the pleopods were detected in *Gnorimosphaeroma oregonensis* (BF) or *Sphaeroma pentodon*. In *G. oregonensis* (EF), however, changes were rather characteristic. Starting at low tide, when the animals were exposed to fresh water, the body fluid concentration was 50 per cent sea water. This concentration did not change until over five hours later, when the salinity of the habitat had reached 42 per cent sea water, at which time the body fluid concentration was 58 per cent sea water. Then, by the time of the extreme high tide, 1¼ hours later, the body fluid concentration had changed again to 70 per cent sea water, while the medium concentration had changed to 65 per cent sea water.

Discussion

Comparative osmoregulatory abilities

Figure 2 shows the 48-hour body fluid concentrations of the experimental animals in the experimental media. It was assumed that all major changes in body fluid concentration had occurred by 48 hours. In hypotonic media, *Sphaeroma pentodon* appears to be a strong regulator, at least in 50 and 75 per cent sea water. There is no apparent reason for the animals to maintain such high body fluid concentration in those salinities when they can live, at least for several days, in fresh water and 25 per cent sea water with (presumably) much lower body fluid concentrations. *Gnorimosphaeroma oregonensis* (BF) has only limited regulation in all media and appears to be the greatest conformer of the group, maintaining a relatively small gradient between its body fluid and the medium in all salinities. *G. oregonensis* (EF) and *G. oregonensis* (FW) are the most able regulators in terms of the ability to maintain their body fluid concentrations relatively constant in hypotonic media. The body fluid concentration differences between the two forms seen in fresh water, 25 per cent sea water, and 50 per cent sea water, are statistically significant ($t = 6.15$, 3.87, and 12.3, respectively, with 11, 10, and 9 degrees of freedom). The ability of *G. oregonensis* (FW) to maintain its body fluid more concentrated in the hypotonic media perhaps represents the major osmoregulatory difference between the two forms. The estuarine form is intermediate between the bay and fresh-water forms in osmoregulatory ability.

Comparing the osmoregulatory abilities of the isopods in this study with those of other crustaceans, a similarity can be seen to species inhabiting similar salinity ranges. From the results of Lockwood and Croghan (1957), it appears that *Mesidotea entomon* is similar in its osmotic regulation to *Gnorimosphaeroma oregonensis*. The former species consists of two "races" which have adapted to brackish- and fresh-water. As in *G. oregonensis* (FW), the fresh-water *M. entomon* is able to live in salinities up to normal sea water. However, unlike *G. oregonensis* (EF), the brackish-water "race" of *M. entomon* cannot live in fresh water. The brackish-water *M. entomon* is thus closer to *G. oregonensis* (BF) and *Sphaeroma pentodon* in its osmoregulatory abilities. However, *M. entomon* does not show the high degree of hypo-osmotic regulation seen in the isopods in the present study. Beadle and Cragg (1940) reported a difference in the ability to retain chloride between the brackish- and fresh-water forms of the amphipod, *Gammarus duebeni*, when placed in distilled water. The fresh-water form retained sufficient chloride to survive for several days in distilled water, whereas the brackish-

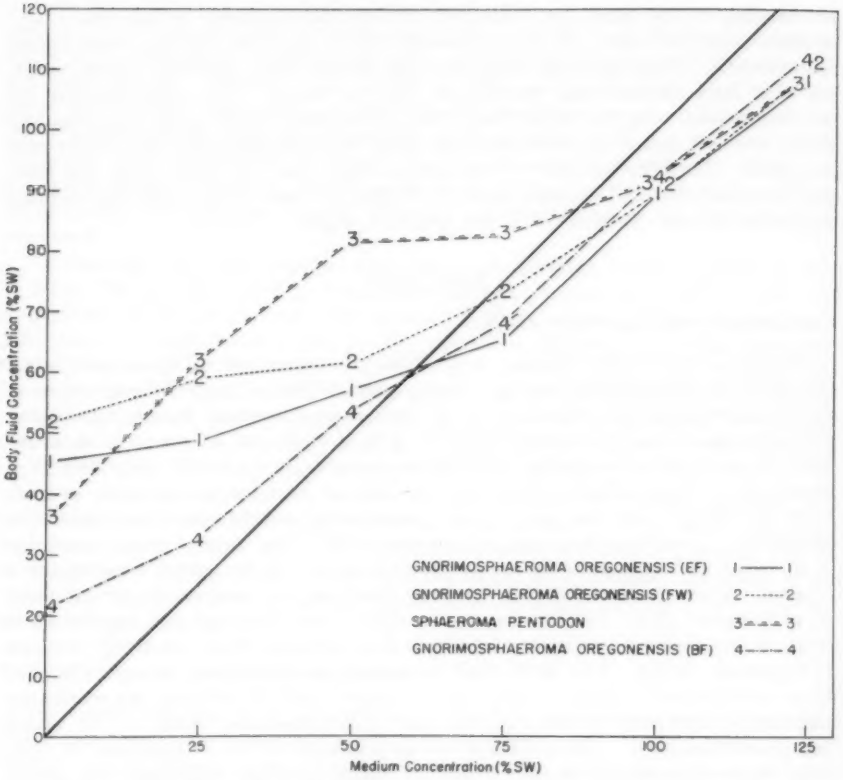

FIGURE 2. Relation of the body fluid concentration to the medium concentration of animals exposed for 48 hours to the experimental salinities.

water form lost chloride and died rapidly in that medium. It appears that the osmoregulatory abilities of the isopods in this study are intermediate between those of one group of crustaceans which can hyper-regulate in dilute sea water, but become isosmotic, or nearly so, in salinities approaching normal sea water (*e.g., Carcinus maenas,* Schlieper, 1929; *Hemigrapsus oregonensis* and *H. nudus,* Jones, 1941) and a second group of crustaceans, which hyper-regulate in dilute sea water and hypo-regulate in salinities approaching normal sea water (*e.g., Heloecius cordiformis,* Edmonds, 1935; *Uca crenulata* and *Pachygrapsus crassipes,* Jones, 1941; *Palaemonetes varians,* Panikkar, 1941; and *Palaemon serratus,* Parry, 1954). All but the last two members of the latter group are primarily semi-terrestrial, which has led Prosser *et al.* (1950a, 1955) to suggest that hypo-osmotic regulation may be associated with the semi-terrestrial habit. The isopods in this study are able to survive for extended periods out of water, but they cannot be classified as semi-terrestrial.

The mechanism of osmoregulation

Although there is little direct evidence elucidating the actual mechanisms of osmotic regulation of the body fluid of the experimental animals, it is possible to make certain hypotheses concerning that phenomenon based on data obtained in the present study and in studies (unpublished) which were made prior to the present study.

a. *Evidence for water movement*

There were no detectable weight changes in the experiments conducted at 16° C., which indicates that there was no net gain or loss of water from the experimental animals' bodies. It is probable that the maintenance of a zero net water flux (that is, no imbalance of the water gain/loss ratio) is dependent upon the ability of the animal to maintain its metabolic rate at a normal level. Duplicate experiments done at 5° C. (see Riegel, 1958) resulted in weight gains by the experimental animals in the dilute salinities and weight losses in the more concentrated salinities. These results may be interpreted as being due to an interference by the low temperature with the normal metabolism of the animals.

b. *Evidence for salt movement*

Since there were no weight changes in the experiments conducted at 16° C., it must be assumed that body fluid concentration changes were due to salt movement. In dilute media (fresh water to 50 per cent sea water), the salt concentration of the body fluid was actively maintained against a gradient. In more concentrated media (75 to 125 per cent sea water), salts were prevented from entering the body (or were eliminated as fast as they came in), since after the initial concentration of the body fluids (generally by 24 hours) the body fluid was maintained hypotonic to the medium. This mechanism could possibly involve, at least in part, an arrest of the mechanism for active salt absorption.

Except for *Gnorimosphaeroma oregonensis* (FW) there was a rapid loss of salts (within three hours) in the more dilute salinities. Whether this loss was due to an active elimination of salts by the animal, thus reducing the concentration gradient between their body fluids and the medium, or a passive loss from the body is not known. There is some evidence suggesting an active elimination of salts in the more dilute salinities, shown especially by *G. oregonensis* (EF) and *Sphaeroma pentodon* after three hours' exposure to fresh water. In those two forms, the body fluid concentrations dropped more rapidly at 16° C. than at 5° C. (see Riegel, 1958).

Whatever the mechanism for the maintenance of the body fluid concentrations in lower salinities, low temperatures interfere with the metabolism of the animals, causing variations in osmoregulation not seen at the higher temperature. In all cases, except *Gnorimosphaeroma oregonensis* (BF) in fresh water, the animals were able to maintain their body fluid concentrations within viable limits after 48 hours' exposure at 16° C. But at 5° C., *G. oregonensis* (BF) was dead after 24 hours' exposure to fresh water and 48 hours' exposure to 25 per cent sea water, and *Sphaeroma pentodon* died after 48 hours' exposure to fresh water. Further, the body fluid concentration of *G. oregonensis* (FW) and *G. oregonensis* (EF) dropped

to subnormal values in fresh water at the lower temperature, but remained within normal limits at the higher temperature.

Wikgren (1953) studied the effect of low temperature on various poikilothermous animals (a crayfish, a lamprey, and a bony fish) and concluded that low temperatures have their chief effect in interfering with the ion-absorbing mechanism of the animals. In the lamprey, urine production was decreased by low temperature, which may have resulted in a weight gain, although Wikgren did not indicate that such was the case. David (1925) performed experiments on the living kidney of the frog, which indicated that that organ's urine diluting and concentrating activity was not affected by temperature. However, Wikgren (1953) recalculated David's data and stated that the diluting capacity of the frog's kidney was reduced by low temperature. Thus, evidence may be inferred from the review by Wikgren (1953) that low temperature adversely affects the ability of cold-blooded animals (at least, cold-blooded vertebrates) to rid the body of water.

The changes in body fluid concentration seen in the present study at 16° C. were undoubtedly due to salt movement. Since there were demonstrated water losses and gains at 5° C., the question arose as to whether the body fluid concentration changes which occurred at that low temperature were due entirely to water movement or were partly due to salt movement.

Because the usual procedures for determining body fluid volume were hardly applicable to animals of such small size as used in this study, that component was estimated in the following manner. Ten animals of each experimental group were weighed, and all the body fluid removed from their bodies that could be collected into capillaries of 1-mm. bore. The animals were then re-weighed. Average collectable body fluid weights as a percentage of total body weight were 9.5, 9.7, 11.1, and 6. 8, respectively, for *Gnorimosphaeroma oregonensis* (FW), *G. oregonensis* (EF), *G. oregonensis* (BF), and *Sphaeroma pentodon.* These values established the minimum possible weight of the body fluid. Ten animals of each experimental group were weighed and dried to constant weight in a calcium chloride desiccator. The average values for total body water as a percentage of the total body weight were 56.5, 55.6, 56.4, and 53.8, respectively, for *G. oregonensis* (FW), *G. oregonensis* (EF), *G. oregonensis* (BF), and *S. pentodon.* These values established the maximum possible weight of the body fluid as a percentage of the total body weight.

Table I compares the calculated and actual dilution and concentration of the body fluids in fresh water and 125 per cent sea water [using a 40-milligram specimen of *Gnorimosphaeroma oregonensis* (EF) as an example] based on estimates of the body fluid weight ranging from ten to 50 per cent of the total body weight. A sample calculation follows: Referring to Table I, it can be seen that a 40-milligram animal, with a body fluid concentration of 50 per cent sea water (column 5), when placed in fresh water would gain 11.3 per cent of its body weight (column 3) after 24 hours. If the weight gain is due entirely to water entry into the body, the incoming water would dilute the body fluids by a factor, X, given by the relation: $X = \frac{\text{wt}_0\ (= \text{original body fluid weight})}{\text{wt}_{24}\ (= \text{24-hour body fluid weight})}$. If the body fluid comprises 50 per cent of the total body weight (column 1), its dilution by the gain of 4.5 milligrams

of water (column 4) would result in a body fluid concentration of $X \cdot 50$ $\left(= \frac{20}{24.5} \cdot 50\right)$, or 40.8 per cent sea water (column 6).

When a 40-milligram animal whose initial body fluid concentration is 50 per cent sea water is placed in 125 per cent sea water, if the body fluid comprises 50 per cent of the total weight, the body fluid would be concentrated by the factor $X\left(= \frac{20}{17.3}\right)$. Thus the body fluid will be concentrated to 57.8 per cent sea water (column 10).

TABLE I

Comparison of actual and calculated concentration and dilution of the body fluids (BF) at 5° C. based on several estimates of the body fluid weight (BF Wt.) as a percentage of total body weight (BW) and assuming the concentration and dilution to be due entirely to water movement*

1 Est. BF Wt. (% BW)	2 Est. BF Wt. (mg.)	3 % BW gain FW	4 BF Wt. after 24 hrs. FW	5 Start. BF conc. (% SW)	6 Calc. BF conc. FW	7 Actual BF conc. 24 hrs. FW	8 % BW loss 125% SW	9 BF Wt. 24 hrs. 125% SW	10 Calc. BF conc. 24 hrs. in 125% SW	11 Actual BF conc. 24 hrs. in 125% SW
50	20	11.3	24.5	50	40.8	42	6.7	17.3	57.8	112
40	16	11.3	20.5	50	39.0	42	6.7	13.3	60.2	112
30	12	11.3	16.5	50	36.4	42	6.7	9.3	64.5	112
20	8	11.3	12.5	50	32.0	42	6.7	5.3	75.4	112
18	7.2	11.3	11.7	50	30.8	42	6.7	4.5	80.0	112
16	6.4	11.3	10.9	50	29.4	42	6.7	3.7	86.5	112
14	5.6	11.3	10.1	50	27.7	42	6.7	2.9	96.6	112
13	5.2	11.3	9.7	50	26.8	42	6.7	2.5	104.0	112
12	4.8	11.3	9.3	50	25.8	42	6.7	2.1	114.3	112
11	4.4	11.3	8.9	50	24.7	42	6.7	1.7	129.4	112
10	4.0	11.3	8.5	50	23.5	42	6.7	1.3	153.8	112

* A 40-mg. specimen of *Gnorimosphaeroma oregonensis* (EF) was used as an example.

From Table I it can be seen that the calculated body fluid concentrations and dilutions in 125 per cent sea water and fresh water, based on estimates of the body fluid weight percentage, do not completely match the actual results. If the estimated body fluid weight of 50 per cent total weight is correct, the calculated dilution in fresh water is close to the actual value. However, the calculated concentration in 125 per cent sea water is much lower than the actual value. If the estimated body fluid weight of 12 per cent total body weight is correct, the calculated body fluid concentration in 125 per cent sea water is close to the actual value, but the calculated body fluid concentration in fresh water is much lower than the actual value. Therefore, it is likely that the actual body fluid weight lies somewhere between 10 and 50 per cent of the total body weight. If a reasonable estimate of 20 to 30 per cent is close to the actual value for the body fluid component of the total body weight, it appears that the actual body fluid concentrations in fresh water and 125 per cent sea water at 5° C. are not due entirely to water movement. That is, it is probable that there is a retention or reabsorption of salts in fresh water and an absorption of salts in 125 per cent sea water.

These results are in general agreement with those of Hukuda (1932) who compared the theoretical and actual change in weight with the observed change in osmotic pressure of the blood in *Portunas puber* when that marine animal was immersed in ⅔ normal sea water. Gross (1957) found in *Emerita analoga* that a weight change of less than two per cent of the body weight resulted in a body fluid concentration change equivalent to 25 per cent sea water. Based on the assumption that osmotically active water comprised 40 per cent of the body weight, he calculated that the weight change, if due entirely to water movement, would have changed the body fluid concentration by less than six per cent.

The estimate of 20 to 30 per cent as the haemolymph component of the body weight in *Gnorimosphaeroma oregonensis* (EF) only partially agrees with similar estimates of that value in other crustaceans. A body fluid value of 50 per cent of body weight was assumed by Lockwood and Croghan (1957) for *Mesidotea entomon*. Similarly, a body fluid of ⅓ body weight was assumed for *Palaemonetes antennarius* by Parry (1957). Gross (1957) made actual calculations of the "solute space" in *Pachygrapsus crassipes* and *Emerita analoga* which were, respectively, 56 and 40 per cent of body weight. However, solute space would be expected to be greater than the body fluid volume and less than the total body water. Approximate measurements of blood volume of various crustaceans have been made using sodium thiocyanate. Nagel (1934) found a blood volume of 37 per cent of body weight in *Carcinus maenas*. Krogh (1939) measured a blood volume of 33 per cent of body weight in *Eriocheir sinensis*. Prosser and Weinstein (1950) measured the body fluid volume of the crayfish, *Orconectes virilis*, obtaining values of 25.6 per cent and 25.1 per cent, respectively, with sodium ferrocyanide and a dye, T-1824. The isopods in the present study seemed to have large amount of exoskeleton relative to soft tissue. This fact was further borne out by the relatively low total water values, and in the writer's opinion, supports the estimate of 20 to 30 per cent of total body weight as the body fluid component.

To summarize, it is probable that the osmoregulatory abilities of the experimental animals include a mechanism for active salt uptake and retention. In the experiments conducted at 16° C., the body fluid concentrations and dilutions were not accompanied by detectable weight losses or gains, suggesting that the concentration and dilution are due to salt movement. Since concentrations and dilutions of the body fluids could not be explained purely on the basis of water movement (weight losses or gains), in experiments conducted at 5° C., there is evidence that concentration changes, especially in the higher salinities (75 to 125 per cent sea water) were also due to salt movement at the low temperature. There is some evidence that the experimental animals actively maintain the normal water content of the body fluid. Though body fluid concentrations were well-marked at 16° C., no weight changes were detected. Rather than propose that no water enters or leaves the bodies of the experimental animals upon exposure to the experimental salinities, it might be more reasonable to assume that the normal body water component is actively maintained by pumping water out as fast as it comes in in hypotonic media and by active water uptake and/or salt elimination in hypertonic media. The fact that weight changes were well-marked in experiments conducted at 5° C. and non-existent in experiments conducted at 16° C. indicates that the mechanism for active maintenance of the water balance of the body is depressed or inactivated by low temperature.

The writer wishes to express his gratitude to Professor Milton A. Miller of the University of California, Davis, for his guidance during the writer's period of graduate study. Appreciation is expressed to Dr. Ralph I. Smith, of the University of California, Berkeley, for suggestions and helpful criticism during the balance of the research embodied in this paper. Sincere thanks go to Dr. A. H. Smith, of the University of California, Davis, for technical aid and advice and critical review of the manuscript, and to the Committee on Research of the University of California for a Graduate Student Research Grant (DG-6) which made a greater part of this work possible. Finally, a special note of thanks to Professor C. Ladd Prosser, of the University of Illinois, who contributed much to the form of the paper presented here by his generous comments and criticism.

Summary

1. Osmoregulatory requirements were analyzed and compared in Menzies' two subspecies of *Gnorimosphaeroma oregonensis* (*G. o. oregonensis* and *G. o. lutea*) and *Sphaeroma pentodon* Richardson.

2. The mechanism of osmoregulation was studied by measuring changes in the total osmotic concentration of the body fluid after three to 48 hours' exposure to various experimental salinities ranging from fresh water to 125 per cent sea water. Changes were also measured in the field during a partial tidal cycle. The principal findings and conclusions are as follows:

a.) The body fluids of the experimental animals became either diluted or concentrated in the experimental salinities. Generally, in more dilute media (50% sea water or less), the body fluids were maintained hypertonic to the medium, while in more concentrated media (75 to 125% sea water), they were usually maintained hypotonic to the medium.

b.) The lack of weight changes in experimental salinities in experiments conducted at 16° C. indicates that dilution and concentration of the body fluid at normal temperatures are caused primarily by salt movement.

c.) Pronounced weight changes that occurred in experiments conducted at 5° C. suggest that the normal water component of the body fluid is actively maintained and that low temperature interferes with this active maintenance, which normally permits excess water to leave the body in diluted media and to enter in more concentrated salinities. However, the fact that the degree of concentration and dilution of the body fluids at the low temperature could not be explained solely on the basis of water movement suggests concurrent salt gains or losses.

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MOTILITY AND POWER DISSIPATION IN FLAGELLATED CELLS, ESPECIALLY CHLAMYDOMONAS[1]

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The energetics of cellular motion have evoked much interest over the past few decades. Muscle, amoeboid cells, and ciliated or flagellated cells have all been studied, but skeletal muscle has received the most attention. This is true partly because the motion of muscle cells can be stopped and started at the will of the experimenter. This fortunate property, absent in amoeboid and ciliated cells, allows the muscle cell to be compared with itself during rest and exercise. Metabolic poisons can be used to stop movement in non-muscular cells, but chemical inhibition is seldom reversible or specific enough for experimental designs as elegant as those possible in studies on muscle.

Recently, genetic mechanisms have been discovered for controlling the motility of certain flagellated cells: the bacterium *Salmonella typhimurium* (Stocker, Zinder and Lederberg, 1953) and the autotrophic green alga, *Chlamydomonas* (Lewin, 1952). Of the two organisms, *Chlamydomonas* has some advantages as an experimental object, since it is nonpathogenic and has simple, well-defined nutrient requirements. By using ultraviolet light, Lewin (1954) has produced several single-locus mutant strains with abnormal flagellar characters, including some which look just like the wild-type strain but do not move their flagella. The paralysis must be related to an abnormality either of flagellar structure or of some other part of the cell. The failure of Mintz and Lewin (1954) to find serological differences between the flagella of normal and paralyzed strains suggests that these flagella may be structurally similar. If this is so, the loss of motility is probably related to a metabolic change elsewhere in the cell. It is now possible by using these algal strains to compare the metabolism of "normal" and "paralyzed" flagellated cells which are presumably alike in other respects. For this comparison it is necessary to assume that a large and definite proportion of the cells in the "normal" culture is motile. An estimate of this proportion, the motility index, will be developed primarily for use in later studies. Its use in this paper will be only to justify the above assumption.

The energetic cost of flagellar motion will be estimated in two ways. One estimate is based on microscopic study of the motile cells, the other on measurements of respiration. The two estimates will be compared.

It is a pleasure to acknowledge the technical assistance of Karl M. Buretz, L. W. Clem, Miss Mary C. Straughn, and Irwin D. Zimmerman; the use of the facilities of the Marine Biological Laboratory, Woods Hole, Massachusetts, in

[1] Aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Delaware, NR 164-280. Technical Report 58-2.

1955; and the helpful comments of Drs. Paul Plesner and Erik Zeuthen, who read the manuscript.

Methods

The marine organisms used in this study, *Amphidinium Klebsi, Carteria* (?) sp. and *Dunaliella* sp., were obtained from Dr. J. H. Ryther at the Woods Hole Oceanographic Institute. The three strains of *Chlamydomonas* came from the Department of Botany, Indiana University (I.U.): they were *C. Moewusii* (+) (I.U. No. 97) herein called "CMW," *C. Moewusii* (+) (Lewin's paralyzed strain No. M 1001; I.U. No. 697) herein called "CMP," and *C. Reinhardi* (+) (I.U. No. 89, Sager and Granick, 1953) herein called "CRW." Marine organisms were studied in filtered, autoclaved Woods Hole sea water and kept on agar slants made with sea water. Fresh-water *Chlamydomonas* was grown and studied in a liquid medium suggested by Fuller [2] which contained

KNO_3, 1 *M*	5.0 ml.
K_2HPO_4, 1 *M*	0.5 ml.
KH_2PO_4, 1 *M*	0.5 ml.
$MgSO_4$, 1 *M*	2.0 ml.
$Ca(NO_3)_2$, 1 *M*	0.25 ml.
"Trace element solution"	1.0 ml.
"Iron solution"	1.0 ml.
Iron-free water to make	1000 ml.

"Trace element solution" contained

H_3BO_3	1.43 g.
$MnSO_4 \cdot H_2O$	1.05 g.
$ZnCl_2$	0.05 g.
$CuSO_4 \cdot 5H_2O$	0.04 g.
$H_2MoO_4 \cdot H_2O$	0.01 g.
Distilled water to make	1000 ml.

"Iron solution" contained

Disodium ethylenediaminetetra-acetate	0.5 g.
$FeSO_4 \cdot 7H_2O$	5.0 g.
Distilled water to make	1000 ml.

The culture vessels were 125-ml. Erlenmeyer flasks containing 50 ml. of medium and 2.5-liter, wide, flat-bottomed culture flasks (like A. H. Thomas No. 4372-F) containing one liter of medium. Air containing 5% CO_2 was bubbled through the larger cultures. The flasks were shaken mechanically to swirl the contents gently. Four fluorescent lamps (type F40T12W or SW) were mounted under a glass-bottomed water thermostat kept at 23° C., in which the larger flasks were immersed to the level of the medium inside. The illumination 2.5 cm. above the bottom of the flasks was about 500 foot-candles as estimated with a photographic exposure meter. The large flasks were inoculated either with 100 ml. of a previous one-liter culture, or with a 50-ml. culture, reared for the purpose in a

[2] R. C. Fuller, personal communication (1955).

small flask. The small flasks were illuminated from above and shaken gently but were not otherwise aerated. One-liter cultures were ready for harvest (about 2×10^6 cells ml.$^{-1}$) in two to four days, depending on the inoculum. The harvest was usually concentrated by gentle centrifugation, and the cells were re-suspended in fresh medium before use.

Motility of whole populations of cells was studied by comparing photomicrographs of samples of cell suspensions. The film (Du Pont Microcopy) was exposed for 8 seconds and developed for maximal contrast with elon-hydroquinone contrast developer (Kodak formula No. D-11). After being processed, the photographs were projected onto a screen for counting those cells which were stationary long enough to form images. Images of moving cells failed to register because of the long photographic exposure. The use of a haemacytometer [3] and a phase-contrast microscope in photography made the counting easier.

This method leads easily to the formulation of a *motility index, M*. A practical definition of M is

$$M = 1 - \frac{n_1 - n_2}{n_3},$$

in which n_1 = the number of cells counted in a defined area of the photograph of a cell suspension, made with a time exposure of 8 seconds;

n_2 = the number of cells in the first photograph whose images fail to appear in the second, otherwise similar, photograph taken one minute later;

n_3 = the number of cells appearing in a photograph of a different drop of the same suspension, in which all the cells are immobilized (*e.g.*, with HCHO or I_2 vapor).

The second measure of motility used here is based on the speed of locomotion of individual motile cells in a drop of a dilute suspension, placed on a slide and covered with a coverglass, at room temperature (21 to 23° C.). The individuals to be studied were selected at random by tracking every cell which crossed a line bisecting the field, for as long as it remained in the field. The image of the cell was projected on to a sheet of paper, using a camera lucida. The path of motion was described by pencil marks indicating the position of the cell every two seconds. A loudly ticking clock or mechanical sounder was found to be essential. The distance travelled by the cell per second was calculated from a summation of the line segments connecting the pencil marks on the sheet, and from the time elapsed between the placement of the first and the last marks. The distances travelled per second by several cells in the same suspension were averaged to estimate the *average speed of locomotion* for the population.

Oxygen consumption was measured at 23° C. by the Warburg method. Each 14-ml. reaction vessel was inclosed in a light-tight cloth bag and contained 2 ml. of a suspension of cells which had been washed by gentle centrifugation (700 × G, 30 seconds) and re-suspended in fresh medium. The manometers were read every 10 minutes. The respiratory rate was found to decline slowly with time, but

[3] A haemacytometer chamber for phase-contrast microscopy is manufactured by the American Optical Co.

not appreciably during the first 90 minutes; the readings during this period were fitted with a straight line by the method of least squares. Respiratory rates were then expressed in μl. of O_2 (S.T.P.) per mg. total nitrogen per hour (Q_{O_2} (N)). Total N was estimated by sulfuric-acid digestion of an aliquot, with three successive additions of H_2O_2, followed by direct Nesslerization and reading of the samples in a Klett-Summerson photoelectric colorimeter (Miller and Miller, 1948).

Results

Degrees of motility in a culture of C. Reinhardi

When samples of a culture of *C. Reinhardi* were observed with the microscope, they were found to contain some stationary cells. Some of these became motile from time to time; at the same time swimming individuals settled down to become members of the stationary group. In general the stationary group seemed to remain constant in size; therefore, in any series of observations the number of *originally* stationary cells becoming active in any time interval may be expected to bear a constant relation to the number of *originally* stationary cells remaining. To test this supposition, a single drop of a culture was photographed repeatedly over a period of several minutes. The photographs were studied, and numbers of originally stationary cells remaining were plotted on a logarithmic scale against time. In one experiment (Fig. 1) the points fell on a straight line for the first

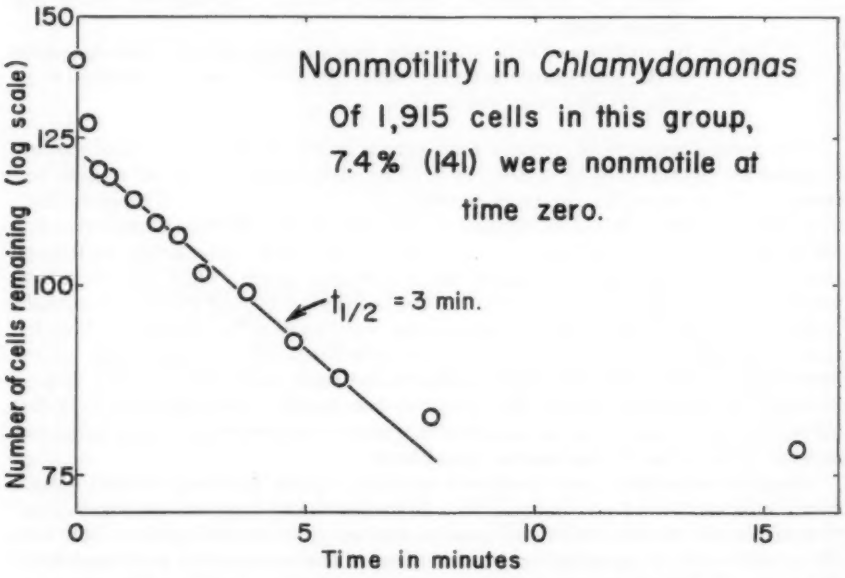

Figure 1. Degrees of motility in a culture of *Chlamydomonas Reinhardi*. For the first 5 minutes many of the originally stationary cells became motile as shown by the points fitted with a straight line. During the first 30 seconds a more active group of cells dominated; a slower, possibly more heterogeneous group dominated after 5 minutes.

five minutes; this supports the hypothesis that a constant proportion of the remaining non-motile cells become active during each time interval. However, the graph also revealed that the entire original population of stationary cells was made up of three classes, according to their rates of decrease. The first two had half-times of one and three minutes, respectively. Cells in the third class, possibly including dead individuals in the culture, failed to move in 16 minutes. For this population the first photograph showed 141 (n_1) stationary cells; in the second photo 23 (n_2) of these particular cells were missing. A photo of a killed sample showed 1915 (n_3) cells. Thus, $M = 0.94$. In general, samples from other cultures gave similar results, except that the "one-minute" class often could not be found.

Locomotion of individual cells of several species

A different quantitative concept of cellular motion results from the detailed observation of single motile cells selected at random from a culture. The path of motion of a flagellated algal cell is a series of straight lines or arcs of large radius. Cells may occasionally change direction abruptly or spin briefly in place as if held by a mucous attachment. In addition, cells which are swimming forward often revolve about an axis parallel to the direction of motion (Brown, 1945) and may oscillate as they swim.

For studies of the velocity of motion, several kinds of elliptical or nearly spherical flagellates were selected. Table I shows the observations and calculations derived from them. The "average radius" is one fourth the sum of length and width. The minimal power dissipation, P, per cell was calculated from Stokes's Law relating to the force, f, needed to propel a sphere through a fluid:

$$f = 6\pi r\eta u,$$

and from the relation

$$P = 10^{17} fu,$$

where

f = force needed to overcome fluid resistance (dyne),
r = average radius of cell (cm.),
η = viscosity of fluid (poise = dyne sec. cm^{-2}),
u = average speed of locomotion (cm. sec.$^{-1}$),
P = power (watt = 10^7 dyne cm. sec.$^{-1}$).

Oxygen consumed by normal and by paralyzed Chlamydomonas

A third aspect of flagellar motion concerns the intensity of metabolism of the flagellated cell. In this study, the oxygen consumption of a population of normal *Chlamydomonas Moewusii* (CMW) was compared with that of the ultraviolet-induced, "paralyzed" mutant (CMP). The mutant cells have flagella but fail to use them; these are held out rigidly almost perpendicular to the main axis of the cell. Occasionally a flagellum showed a little motion at its tip, but this hardly ever caused the whole cell to move.

The figures in Table II are based on 12 reaction vessels for CMW and 13 for CMP. In preparation for each experiment the cells of the two strains were reared

TABLE I

Minimal power output of selected flagellated cells

Species	Size: Aver. radius 10^{-4} cm.	Size: Length / Width	Medium	Aver. velocity 10^{-2} cm. sec.$^{-1}$	Min. power output/cell, 10^{-10} watt
Amphidinium Klebsi	7.98 (11)	1.30	Sea water	0.739 (11)	7.5
Carteria (?) sp.	6.54 (9)	1.67	Sea water	1.25 (10)	17
Chlamydomonas Moewusii (CMW)	5.54 (11)	1.48	Fresh water	1.28 (16)	15
Dunaliella sp.	4.40 (9)	1.35	Sea water	2.26 (9)	38
Chlam. Reinhardi (CRW)	3.26 (100)	1.08	Fresh water	0.828 (100)	3.9

Numbers of individuals studied are in parentheses. Viscosities (corrected for density) used in calculations were: sea water, 0.965 cp (estimated from Miyake and Koizumi, 1948); fresh water, 0.931 cp.

in one-liter cultures under identical conditions. In each experiment the oxygen consumption of the paralyzed cells was less than that of the normal cells when expressed in terms of total cellular nitrogen.

DISCUSSION

The quantitative description of cellular motility will be discussed before considering the energy required for flagellar motion. This study presents two quantitative methods of studying locomotion in populations of flagellated cells.

The *motility index* (photographic method) can be used for distinguishing the behavior of cell populations exposed to varying experimental treatments. It may prove helpful in pharmacologic and toxicologic studies on suspensions of algae, protozoa, bacteria, or sperm cells; these forms may offer the experimenter advantages over larger and more expensive animal subjects. Compared with other proposed estimates of the proportion of non-motile cells in a microscopic field (Emmens, 1947; White, 1954) it would appear to avoid certain subjective errors in sampling and counting, and to minimize the error due to the inclusion of cells

TABLE II

Oxygen consumption (Q_{O_2} (N)) of normal and paralyzed Chlamydomonas Moewusii (paired comparison)

Experiment no.	CMW	CMP	Difference (CMW-CMP)
6-27-57	1.24	1.14	0.10
7- 1-57	1.36	1.24	0.12
7-22-57	1.31	0.79	0.52
8- 3-57	1.19	1.14	0.05
8- 5-57	2.33	2.06	0.27
8- 7-57	2.00	1.72	0.28
Mean difference and its standard error ($n = 6$)			0.223 ± 0.0706

which may stop for momentary "rest" periods. It cannot distinguish degrees of impairment of locomotion.

The *average speed of locomotion* appears to be valuable for distinguishing populations of cells which show normal speeds of locomotion from populations with impaired locomotion. It takes no notice of non-motile cells, and thus becomes most useful in estimating the degree of motility in cultures where the motility index is high. It is similar in principle to one devised by Baker, Cragle, Salisbury and Van Demark (1957) who measured the time required for 100 free-swimming sperm cells to pass through a segment of a plane. Their method, which seems admirably suited to cells displaying the sperm type of locomotion, has the advantage of presenting the result of an experiment immediately without waiting for photographic processing. The decision to use a given method will rest partly upon the extent to which its assumptions are fulfilled by the swimming habits of the organism. The method described here is of special value, since from it can be derived an estimate of the external work done by the motile cells in the population.

The estimates of power dissipation in Table I are certainly low, because the premises on which they are based all tend to reduce the estimates. It is supposed, for example, that the cell's internal energetic conversions are 100% efficient. The other assumptions, each known to be false to some extent, are: that there are no degrees of motion other than uniform in a straight line (contradicted by Brown, 1945, and others), that the cell is a sphere (contradicted in Table III), and that

TABLE III

Estimates of size of Chlamydomonas

Strain.....	CRW	CMW	CMP
Length, μ	6.49 ± 0.14	7.64 ± 0.17	7.41 ± 0.14
Width, μ	5.24 ± 0.17	5.62 ± 0.14	4.92 ± 0.10

The "±" sign is inserted between the mean and its standard error. Fifty cells of a single culture of each strain were measured.

the frictional drag of the flagellum, apparently of major importance in the locomotion of sea urchin sperm (Gray and Hancock, 1955), is negligible in *Chlamydomonas*. Excepting *C. Reinhardi*, the smaller flagellates travelled faster and displayed a higher power output than the larger cells. Whether this difference is related to a greater metabolic rate of the smaller cells has not been determined.

When normal and paralyzed *C. Moewusii* were reared and studied under the same conditions in several successive experiments, the paralyzed cells (CMP) always consumed less oxygen than did the wild-type, motile cells (CMW). The average difference in Q_{O_2} (N) was about 14% of that of the normal cells, and was found to be statistically significant (t-test, $n = 6$) at the 5% level, but not at the 2% level. It must be assumed that the proportion of dead cells in the CMP culture is no greater than in the CMW culture. In interpreting this difference, certain other features of the two strains should be borne in mind.

Ocular micrometer measurements showed that although CMW and CMP are of equal length, the paralyzed cells are, on the average, a little more slender than

the motile ones (Table III). Thus, a paralyzed cell's surface-to-volume ratio is slightly greater than that of a normal cell. From the size of this difference alone one would expect the Q_{O_2} (N) of the paralyzed strain to be a little greater than that of the normal strain; it appears in fact to be less. The single mutation which resulted in paralysis of the flagella may have had other expressions, possibly involving alterations in the efficiency of biochemical pathways of metabolism. In summary, the physiologic differences between the two strains may be much greater than appeared at first. In ignorance of the magnitudes of these possible factors, it is tempting to suggest that the difference in oxygen consumption is actually related to the state of motility of the cell, but a cautious attitude seems desirable.

As a partial test of this relationship, we may now compare the two available estimates of the energy required for motility. One of these (Table I) states that *C. Moewusii* dissipates at least 10^{-15} watt per cell in overcoming the frictional losses in water. The other estimate is derived from the difference in Q_{O_2} (N) between the normal and paralyzed strains, which is 0.22 μl. hr.$^{-1}$ (mg. N)$^{-1}$. If we suppose that the exclusion of light from the Warburg vessel does not affect motility (Lewin, 1953), the two figures are comparable; the latter figure can then be transformed to watts per cell by making the following reasonable assumptions:

1. The consumption of 1 μl. of O_2 releases about 4.8×10^{-3} calorie or 5.58×10^{-6} watt hour.
2. A CMW cell contains 2.65×10^{-9} mg. N (estimated from cell counts and N determinations on a single culture at the time of harvest).
3. The motility index in the CMW culture is high.

The observed difference in the rate of oxygen consumption thus corresponds to a difference in power dissipation of 3.3×10^{-15} watt per cell. Rothschild's (1953) reworking of Taylor's figures gives estimates close to these for the minimal energy dissipated by bull sperm: for two kinds of assumptions, 3.74×10^{-14} watt and 2.04×10^{-15} watt per cell. In our comparison, the efficiency of conversion of chemical to mechanical energy is not taken into account. The closeness of the two estimates derived in this paper suggests that the lower rate of oxygen consumption of paralyzed cells may be correlated with their loss of motility.

Summary

1. The paper describes a method for estimating the minimal power output of individual, nearly spherical, flagellated cells. A comparison of 5 species of green flagellates suggests no relationship between size and power dissipation (Table I).

2. A simple photographic method for estimating the fraction of motile organisms in a culture is described.

3. Cultured populations of *Chlamydomonas Reinhardi* may contain two or more distinct groups of cells with different degrees of motility (Fig. 1).

4. The motile, "wild-type" *C. Moewusii* consumed 1.57 μl. O_2 (S.T.P.) per hour per mg. total N.

5. A paralyzed mutant strain of the same species consumed 14% less oxygen than the wild type. The extra oxygen consumed by the motile strain is commensurate with its estimated minimal power output.

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CONSEQUENCES OF UNILATERAL ULTRAVIOLET RADIATION OF SEA URCHIN EGGS[1]

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The suppression of the elevation of the fertilization membrane on the half of a sea urchin egg which directly receives high doses of ultraviolet light has been described by Reed (1943) and Spikes (1944). The experiments reported herein are an examination of the consequences of unilateral U.V. irradiation of the sea urchin egg in terms of changes in cell morphology with dose, the physical state of the cytoplasm, the effects of time and temperature, and the effects on subsequent cell division. Particular attention is directed toward observations on hyaline layer formation, local gelation, and excentric formation of the mitotic figure.

Materials and Methods

Gametes were obtained from the sea urchin *Strongylocentrotus purpuratus* by injection with 0.5 *M* KCl. The groups of eggs selected were more than 99% fertilizable, were free from visible abnormalities, yielded symmetrical fertilization membranes, and showed little distortion when the lifting of the fertilization membrane began. The pattern of morphological changes at different doses was confirmed with suitable eggs obtained from a single female of the related species *Strongylocentrotus franciscanus,* which has larger eggs with less yolk.

The ultraviolet source was an Electrotherapy Products Corp. low pressure mercury vapor lamp, which produces approximately 95% of its U.V. energy in a 2537 Å band. The intensity was measured with a Hanoviameter.

In some experiments the eggs were centrifuged in a Servall refrigerated angle-head centrifuge, either in sea water or in a sucrose gradient formed by layering sea water over 0.88 *M* sucrose.

Unless otherwise noted, all experiments were carried out in 1 cm. deep, filtered sea water at 17.5 ± 0.1° C. Artificial calcium-free sea water was prepared according to the formula of Moore (1956).

Clarification of terminology

In order to describe concisely and accurately the changes associated with unilateral irradiation of the strongly-absorbing egg certain special terms must be defined. The *directly-irradiated hemisphere* is the surface of the egg which faces

[1] Supported by grants from the American Cancer Society and the Office of Naval Research awarded to Dr. Daniel Mazia.

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the U.V. lamp. The *shaded hemisphere* is the surface which does not face the lamp, and, hence, is shaded by the cytoplasm. The *shaded-irradiated axis* is an imaginary line drawn between the *poles* or centers of these two hemispheres. *Unilateral membranes* are fertilization membranes which lift off the egg on the shaded hemisphere only. All drawings and photographs except Figures 1 and 8 have been mounted with the shaded pole facing the top of the page.

Results

When eggs were irradiated with large doses of U.V. and then fertilized, the height of the fertilization membrane and the hyaline layer on the directly-irradiated hemisphere was reduced. Sufficiently large doses unilaterally inhibited the formation of these membranes entirely.

The dose required to produce a definable level of effect varied by as much as a factor of three between the most sensitive and the most resistant groups of eggs. Nevertheless, the ratio of doses necessary to produce two definable effects on the majority of eggs in a population appeared to be constant even in the extreme cases. The data presented represent the most frequently encountered dose relations.

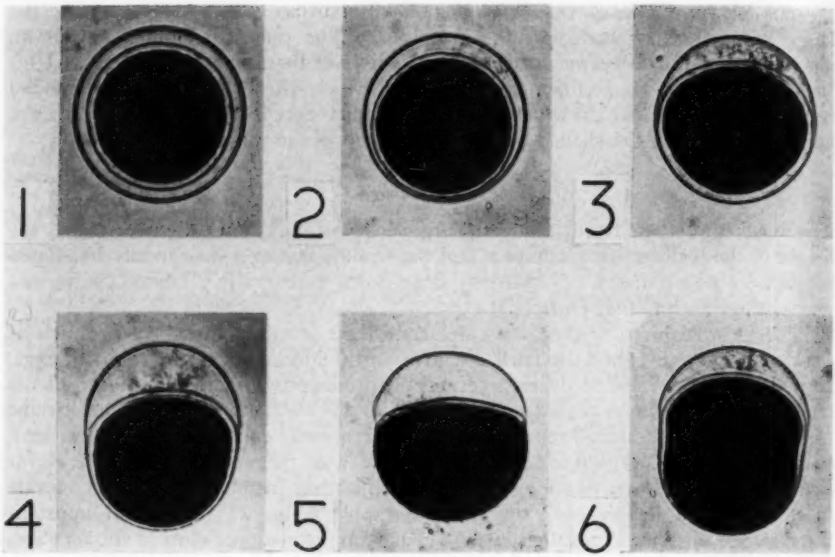
Less than 1600 ergs/mm.^{2} did not interfere with the normal membrane elevation. When the dose was increased the fertilization membranes did not elevate to their normal height over the irradiated pole (Fig. 2). Doses of approximately 2800 ergs/mm.^{2} resulted in the almost complete suppression of the fertilization membrane over a small area, but the hyaline layer differentiated over the entire surface. When the dose was increased to 4800 ergs/mm.^{2} the fertilization membrane covered only one hemisphere, while the hyaline layer appeared normal (Fig. 3). With slightly higher doses a reduction in the thickness of the hyaline layer was sometimes found (Fig. 4). With doses above 7200 ergs/mm.^{2} the hyaline layer could be distinguished only slightly beyond the cell equator (Fig. 5). No further changes in the pattern of membrane elevation were noted at increased doses up to the range of 40,000 to 50,000 ergs/mm.^{2} At this dose level partial cytolysis often occurred immediately on the directly-irradiated hemisphere, and complete cytolysis usually followed after standing or at fertilization.

Identification of the inhibited surface

A simple experimental procedure was devised to demonstrate that the irradiated surface was in fact the one that showed inhibition at fertilization. Stationary eggs were irradiated from above with 7200 ergs/mm.^{2} in a large petri dish on a microscope stage and observed as sperm were carefully added. In four experiments there was no detectable net rotation of any of the eggs in the field of a low power objective. By careful focussing it was established that the fertilization membranes first encircled the lower hemispheres which had been shaded by cytoplasm. As the membranes raised further the eggs rolled over and came to rest on their sides revealing total suppression of membrane elevation on the irradiated hemispheres.

Relationship to time and temperature

Eggs were fertilized at regular intervals from a few seconds after irradiation to as much as twelve hours later without any visible changes in the unilateral



FIGURES 1 to 6

Photomicrographs of sea urchin eggs showing different degrees of suppression of the fertilization reaction when irradiated with increasing doses of U.V. from the direction of the bottom of the page.

FIGURE 1. Control.

FIGURE 2. Reduction of the height of the fertilization membrane.

FIGURE 3. Complete suppression of the elevation of the fertilization membrane on the directly-irradiated hemisphere.

FIGURE 4. Reduction of the height of the hyaline layer.

FIGURE 5. Complete suppression of both fertilization membrane elevation and the hyaline layer differentiation on one hemisphere.

FIGURE 6. Later swelling of the initially flattened shaded hemisphere of an egg similar to Figure 4.

fertilization reaction. In five separate experiments there was no increase or decrease in the inhibited area with time. In general, the irradiated eggs cytolyzed sooner than the controls, but in most experiments both the irradiated and the control eggs became unfertilizable at approximately the same time, even with very high concentrations of sperm.

Attempts were made to re-fertilize eggs which had been fertilized but did not completely differentiate the hyaline layer. The simple addition of viable sperm did not cause re-fertilization at any time up to 28 hours after irradiation. The sperm were observed to accumulate in the egg jelly which adhered to the irradiated hemisphere in each of these experiments.

Irradiating eggs from the same females at 18 and 8° C. with various doses revealed that there were no differences in sensitivity at the two temperatures.

Changes in morphology and physical state of the cytoplasm

The progressive dose-dependent suppression on the elevation of the fertilization membrane and the differentiation of the hyaline layer have already been described. Sometimes at high doses the fertilization membrane was elevated to an abnormal height above the shaded pole and the cytoplasm under it was considerably flattened (Figs. 4 and 5). A large amount of particulate matter, possibly cortical granule materials, was found in the perivitelline space under these conditions. The amount of this material was apparently greater at all doses than in the controls.

After flattening, the cytoplasm under the unilateral membranes sometimes swelled and reduced the thickness of the perivitelline space (Fig. 6). In some cases the thickness was less than the controls. Under these conditions there was a constriction around the cell at the equator where the fertilization membrane met the hyaline layer (Fig. 6).

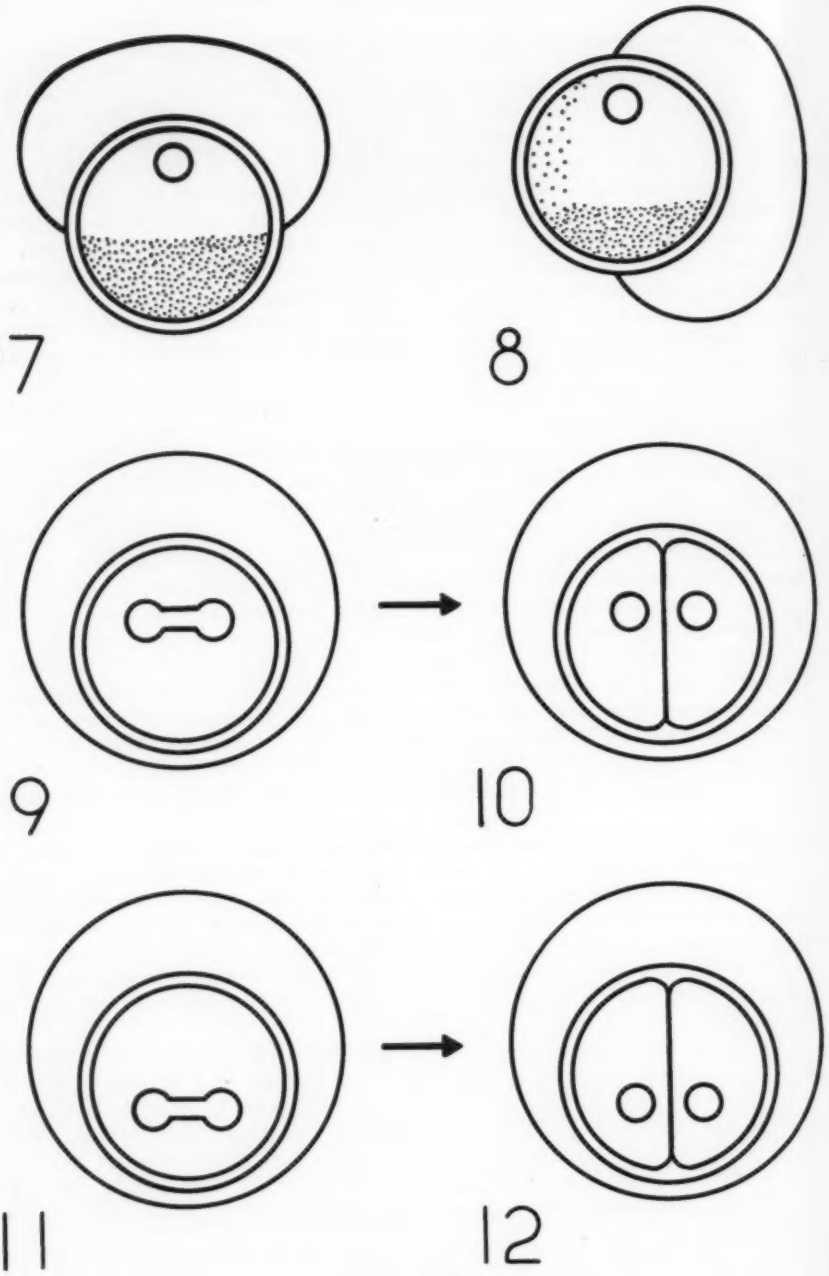
There were no cases of membrane elevation activation by U.V. at any dose in any of the experiments.

Unfertilized irradiated eggs were centrifuged for ten minutes at approximately 12,000 g in a sucrose gradient. In two such experiments 90% of the eggs stratified with the center of the light pole (identified by an oil cap over a clear region of cytoplasm) in the center of the shaded hemisphere (identified by subsequent fertilization) (Fig. 7). Almost all of the remaining 10% had an asymmetry of less than 30° between the light-heavy and the shaded-irradiated axis. A very small fraction of a per cent were 30 to 90° off center, and no cases were found in which the shaded pole appeared to have a greater density than the irradiated one.

When unfertilized eggs were placed in 70% sea water after irradiation they swelled on one pole only, giving the eggs a somewhat pear-shaped appearance. Standing in this hypotonic medium for several hours did not result in any further changes in shape. The treated eggs were fertilized to establish that the shaded pole was the swollen one. Hence, while both unirradiated eggs and the shaded side of an irradiated one swell in 70% sea water, the directly irradiated surface does not.

Irradiated eggs placed in 70% sea water had a dense darkened area near the irradiated pole, a somewhat less dense region at the shaded pole, and a lighter less granular region near the equator. Occasionally this pattern appeared in eggs kept in normal sea water and seemed to be accompanied by a slight enlargement of the shaded hemisphere. With doses of the order of 40,000 ergs/mm.^{2} a large blister of non-granular material formed on the irradiated pole when the eggs were placed in the hypotonic sea water. With slightly higher doses these blisters appeared spontaneously.

Irradiated eggs were centrifuged at approximately 12,000 g in sufficiently dense suspensions that some of the eggs were confined in a random orientation with respect to their light-heavy axes. Some of these cells showed stratification only on the shaded side, which was identified by subsequent fertilization. When the direction of centrifugation was perpendicular to the shaded-irradiated axis there was a narrow region near the irradiated surface with a very high gel strength that resisted stratification when the central cytoplasm and the shaded side stratified (Fig. 8).



FIGURES 7–12

Eggs irradiated after equilibration in calcium-free artificial sea water and fertilized immediately when returned to normal sea water showed the same degree of inhibition as eggs irradiated in normal sea water.

Mitotic abnormalities

Cells irradiated at doses that inhibited the full differentiation of the hyaline layer seldom divided. At lower doses some or all of the eggs would divide several times and sometimes form apparently normal swimming blastulae. Gastrulation was usually abnormal. In some experiments even the first division was abnormal.

A systematic group of abnormalities occurred as a result of the mitotic figure failing to migrate to the center of the egg. The nucleus of the unfertilized egg is excentrically located, and in normal division the mitotic apparatus is *positioned* approximately in the center of the cell. The position of the furrow is determined by the plane formerly occupied by the metaphase plate both in normal cells and these abnormal cells.

When the mitotic figure located in either hemisphere was oriented perpendicular to the shaded-irradiated axis, the furrow formed along that axis and the egg cleaved into two equal-sized blastomeres (Figs. 9 to 12).

When the mitotic figure was oriented parallel to the shaded-irradiated axis in either hemisphere, the furrow formed perpendicular to the axis and the sizes of the resulting blastomeres were quite different (Figs. 13 to 16).

Variable results were observed when the mitotic figure was formed with other orientations with respect to the shaded-irradiated axis (Figs. 17 and 18).

Excentric spindles were also found in eggs which were irradiated during the early part of the first mitotic cycle with comparatively low doses of U.V. The blastomeres in such experiments were always equal in size.

Whenever the mitotic apparatus was excentric the furrow formed first on the surface that was closest to the spindle. At later stages of cytokinesis the furrow on the near side would always be deeper than the furrow on the far side. In some cases the furrow actually passed through the spindle before the first indentation occurred on the far side of the cell.

Discussion

The progressive unilateral inhibition of the fertilization reaction has been described in terms of the U.V. doses required to produce different degrees of inhibi-

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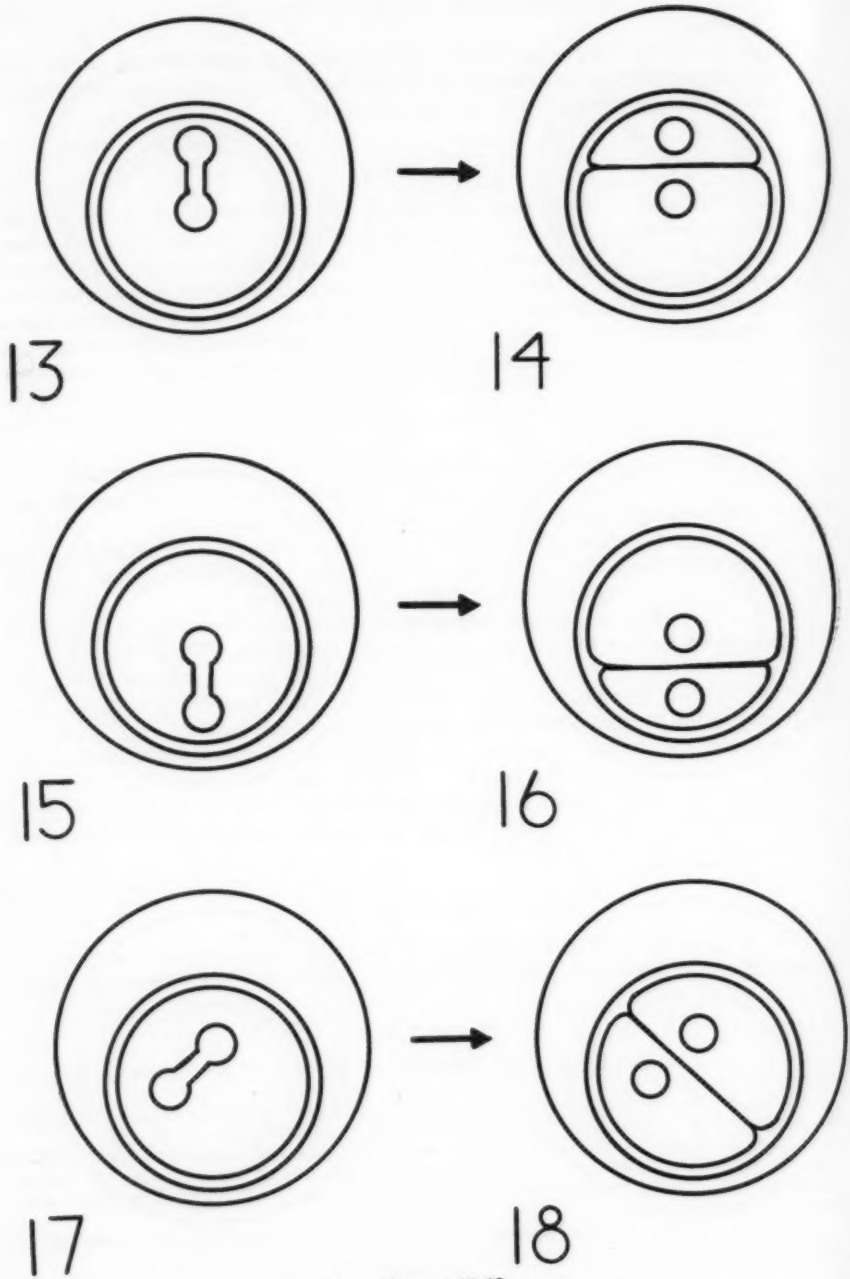
Figures 7 to 18

Schematic drawings of eggs irradiated from the direction of the bottom of the page (except Fig. 8); refer to text for explanation.

Figure 7. Egg centrifuged in a sucrose gradient and then fertilized. Stratification direction indicates that the irradiated pole was heavier than the shaded pole.

Figure 8. Egg irradiated from the left side of the page and centrifuged while confined with the shaded-irradiated axis perpendicular to the direction of centrifugation. A narrow region near the surface of the irradiated hemisphere resisted stratification indicating a local increase in gel strength.

Figures 9 to 12. Division patterns of cells with spindles oriented perpendicular to the shaded-irradiated axis.



FIGURES 13–18

tion of both the elevation of the fertilization membrane and the differentiation of the hyaline layer. Hyaline layer differentiation is less sensitive to U.V. than fertilization membrane elevation; however, it may be suppressed completely on the directly-irradiated hemisphere with high doses. The inhibition of the elevation of the fertilization membrane has been described previously by Reed (1943) and Spikes (1944).

By means of local dye experiments Spikes (1944) was able to demonstrate that the directly-irradiated hemisphere is the site of inhibition. His findings have been reconfirmed with the direct observations of undisturbed eggs reported herein. Giese (1947) has shown that the sea urchin egg strongly absorbs or scatters 2537 Å U.V. light. Harvey and Lavin's (1944) U.V. photomicrographs also indicate that a considerable amount of the light is absorbed in sea urchin eggs of another genus. Since the shaded pole is not inhibited even at very high doses, it may be concluded that the transmission of the cytoplasm is too low to allow the necessary energy to reach the sensitive sites on the shaded side of the egg.

The demonstration that there was no spreading of the damaged area with time indicates that the U.V. action is relatively direct, and, in particular, that there is no secondary effect of "diffusible poisons." There was no recovery with time; hence, the damage seems to be irreversible by any metabolic mechanism. Since the degree of injury did not decrease with time, and a diffusible toxic product would be expected to decrease in local concentration, this observation provides additional evidence against the action of such substances.

The sensitivity was the same at 8 and 18° C.

Direct photochemical action has been shown repeatedly to have a Q_{10} of approximately 1. Therefore, insofar as visually equivalent degrees of damage may be used as a measure of the rate of damage, it appears that the injury results from direct photochemical action. The time and temperature relations together offer evidence that the effect is localized and that there is a lack of intermediary toxic products.

The observation that the inhibited surface could not be re-fertilized by the addition of fresh sperm could be interpreted in two ways: either the U.V. damage rendered it unfertilizable or some of the steps of the fertilization reaction occurred on this side when the egg was initially fertilized. If some substances necessary for the initial steps of the reaction had been used up the sperm could not initiate a response later. A pronounced green Becke line appears in the out-of-focus image of the damaged hemisphere of heavily irradiated eggs after fertilization. This change is probably similar to the dark-field changes which have been observed prior to membrane elevation (Runnström, 1928; Rothschild and Swann, 1949) and indicates that some step in the fertilization reaction has taken place.

Two types of evidence for local gelation in the irradiated hemispheres were obtained: first, that swelling in 70% sea water was confined to the shaded pole, and, second, that a narrow band near the irradiated surface resisted stratification with centrifugation when the rest of the cytoplasm stratified. Reed (1948) found

FIGURES 13 to 16. Division patterns of cells with spindles oriented parallel to the shaded-irradiated axis.

FIGURES 17 and 18. An example of one of several division patterns obtained when the spindles have intermediate angular orientations.

that moderate doses of unilateral U.V. did not change the permeability of the egg to a large variety of solutions. Although no measurements were made, he discussed possible differences at higher doses and proposed that some sort of gelation occurred on the basis that vacuoles were formed in the irradiated pole. Spikes (1944) also proposed that gelation occurred, because he found that while normal eggs only swelled in 50% sea water, irradiated ones lysed on the irradiated side.

Spikes' data might also be interpreted as indicating either that the surface of the shaded hemisphere was weakened or that the osmotically inert volume had been increased permitting greater than normal swelling followed by lysis. The observation of the large amounts of granular material released into the perivitelline space at the shaded pole suggests the weakening either of the cell membrane or of some other surface structure. The flattening of the shaded pole at fertilization at high doses seems to fit either hypothesis, although an enhancement of the vigor of the fertilization reaction would yield the same pattern. It would not be unreasonable to suppose that U.V. damage could affect both the surface strength and the osmotically inert volume, perhaps by a common mechanism.

The observation that eggs irradiated in calcium-free sea water showed the same degree of damage as eggs in normal sea water cannot be interpreted directly in terms of the often demonstrated role of calcium in gelation (Heilbrunn, 1952). First, the eggs had to be fertilized in normal sea water since fertilization will not occur in the absence of external calcium ion; hence, new calcium may have been introduced before the damage was measured. Second, since Heilbrunn and his co-workers have shown that U.V. causes solation in low doses and gelation in high doses, it is quite possible that the calcium ion left in the egg after treatment with calcium-free sea water shifts between the less and more heavily damaged portions of the cytoplasm. The second possibility is quite attractive, since it would provide a mechanism for an increase in osmotically inert volume in the less damaged hemisphere and introduces the possibility that the surface on the shaded side might be weakened by small amounts of U.V. penetrating the cytoplasm to cause solation.

Spikes (1944) reported that in *Lytechinus pictus* furrow formation almost always occurs along the shaded-irradiated axis. Clearly this is not the case in the *Strongylocentrotus purpuratus* used in these experiments; cleavage may take place with any orientation. Successful cleavage with the furrow passing through the irradiated portion of the egg indicates either that the furrowing strength exceeds the resistance of the radiation-induced gel or that the gel is solated in the course of cytokinesis.

Cleavage into equal or unequal sized blastomeres is determined by the orientation of the spindle with respect to the shaded-irradiated axis. It occurs because the mitotic figure remains centered around the original location of the nucleus. The nucleus is excentrically located in unfertilized eggs of this species. When the axis of the mitotic figure is perpendicular to the shaded-irradiated axis the blastomeres are equal in size. Where the axes are parallel the blastomeres are unequally sized. In intermediate angular orientations the results are variable. While both parallel and perpendicular orientations can occur when the mitotic figure is located in either the shaded or irradiated hemisphere, mitotic figures near the equator seem to be restricted to intermediate angular orientations. It is clear that the migration of the nucleus to its normal central position is inhibited. An

increase in cytoplasmic viscosity would provide a plausible explanation for this failure of migration.

It is a great pleasure to acknowledge my gratitude to Professor Daniel Mazia for his helpful advice and encouragement during the course of this work. I also wish to thank Professors J. E. Gullberg, L. V. Heilbrunn and C. B. Metz for their valuable comments about the results, and Mr. Fred Burnet for his skillful preparation of the drawings.

Summary

1. The progressive dose-dependent inhibition of the fertilization reaction on the directly-irradiated hemisphere of the unilaterally U.V.-irradiated sea urchin egg has been described in terms of changes in the ability to elevate the fertilization membrane and to differentiate the hyaline layer.

2. Membrane elevation was not activated by 2537 Å U.V. light.

3. No spreading of the extent of injury or recovery was found with time; and no temperature sensitivity differences were found; hence, the injury appeared to be the result of direct photochemical action.

4. The irradiated hemisphere of the fertilized egg maintained its jelly for considerable periods of time.

5. Evidence was obtained showing partial gelation of the irradiated hemisphere and suggesting that the gelled cytoplasm had a higher density than the rest of the egg. Irradiation in calcium-free sea water did not change the degree of damage observed after fertilization in normal sea water.

6. The behavior of the cytoplasm of the shaded hemisphere at fertilization suggested either that the surface structure was damaged or that the osmotically inert volume had been increased.

7. Unilateral irradiation caused excentric spindle formation which resulted in equal sized blastomeres if the spindle axis was perpendicular to the axis of irradiation and unequal sized blastomeres if the axes were parallel.

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THE ROLE OF THE INITIATOR CELL IN SLIME MOLD AGGREGATION [1]

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Previous studies of slime mold aggregation (Sussman and Noel, 1952) had shown that the number of aggregative centers is linearly related to the number of cells present and, further, that centers are distributed in accord with the Poisson series among small, replicate population samples. These and supporting data were considered to dictate the existence of specially endowed individuals termed "initiator cells," each of which could evoke the aggregative response by its neighbors, the "responder cells." Recently a distinctive cell type was detected by morphological criteria in *Dictyostelium discoideum* Raper and evidence was presented in support of the contention that cells of this type are in fact the initiators of aggregation (Ennis and Sussman, 1958a, 1958b; Sussman, 1958). The distinctive individuals, termed I-cells, are much larger than the remainder of the population (R-cells), the difference amounting to 2–3-fold in diameter, 3–10-fold in area. They are much flatter and more heavily granulated and vacuolated. In contrast to the R-cells which move sluggishly, the I-cells are highly motile and extensive lobopodia and filopodia are seen to protrude constantly and explosively. Figure 1 presents histograms to illustrate the size differences. Two modes are apparent without overlap.

The evidence (Ennis and Sussman, 1958b) supporting the candidacy of the I-cells for the appellation of "initiator" is summarized below:

a) The ratio of I-cells to R-cells remained constant during the pre-aggregative period at 1:1940. This figure agrees closely with the ratio of centers formed to cells present at optimal density (1:2200).

b) A high correlation was encountered between the positions of I-cells and of subsequently formed aggregative centers.

c) The appearance of centers among small, replicate population samples was correlated (perfectly in one experimental series and almost perfectly in another) with the previously determined incidence of I-cells. That is, centers appeared in samples containing I-cells; none appeared in samples without I-cells.

d) Removal of I-cells at an early enough time prevented subsequent center formation.

[1] This work was supported by grants from the National Cancer Institute and the Office of Naval Research.

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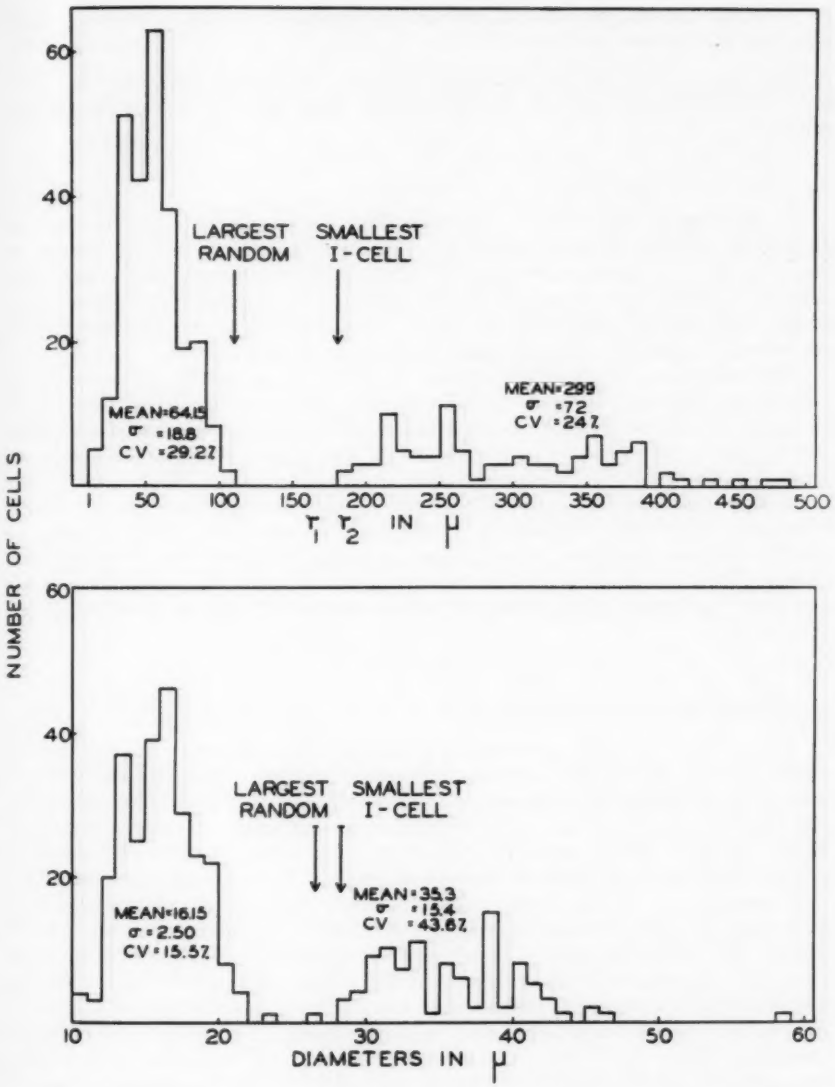

FIGURE 1. Histograms of mean diameters and products of major and minor radii. I-cells were detected under 100× and confirmed under 440× as described in the Methods section. As controls, myxamoebae were chosen at random for micrometric determinations.

e) Micromanipulation of I-cells to test areas caused the induction of aggregates within the test populations, whereas movement of R-cells at the same stage of development did not.

The data to be presented provide subsidiary support for the contention that the I-cells are the initiator cells and throw light upon their role in the aggregative sequence.

Methods

D. discoideum, strain NC-4 wild type, was grown on SM agar medium in association with *Aerobacter aerogenes* (Sussman and Noel, 1952). After 44 hours at 22° C., the myxamoebae had attained the stationary phase and were harvested, washed by differential centrifugation, and dispensed on washed agar plates (Sussman and Noel, 1952). Under these conditions the myxamoebae do not increase in number and aggregate and fruit in normal fashion.

The procedure for I-cell identification has been previously given in detail (Ennis and Sussman, 1958b). Initial recognition is accomplished at 100 × magnification by size and flatness. Examination at 440 × reveals the great pseudopodial activity, high rate of protoplasmic streaming, and granulation, and thereby confirms the diagnosis. A cautionary note is appended here. Occasionally, one encounters moribund cells which typically attain enormous size before lysing. However, these cells are perfectly round and hemispherical. They display no motility and have lost their granules and vacuoles. After one has seen an I-cell, there is no chance of confusing the two types and in any case, moribund cells are extremely rare under the conditions of preparation and incubation described above.

Results

A. Time-lapse studies of aggregation

As mentioned, a high correlation was shown to exist between the positions of I-cells and of subsequently formed aggregative centers. In these experiments, washed myxamoebae were dispensed on washed agar at a population density of 200 cells/mm.^{2}, optimal for center formation in this strain (Sussman and Noel, 1952). After 8 hours' incubation at 22° C., low power fields were chosen at random and fixed in position on microscope stages. Those found to contain I-cells were retained for further study. Camera lucida drawings or photomicrographs were made at intervals until aggregation had begun and the centers were established. In 50% of the fields, a center formed precisely at the position of the I-cell. (Since in this stock, the ratio of centers formed to cells present is 1:2200, the random chance of predicting that a center would form at a particular cell is 0.05%.) In 30% of the fields a center formed near the I-cell. No centers formed in the remaining 20%. In contrast, the incidence of centers in randomly chosen fields, not examined for the presence of I-cells, was 25%. Thus the over-all chance of a center appearing in a field containing an I-cell was three times greater than random. The numerical data, given in detail elsewhere (Ennis and Sussman, 1958b), are here amplified by time-lapse series of camera lucida drawings and photomicrographs.

Figure 2 illustrates the sequence of events when the center formed at the I-cell. The first overt sign of impending aggregation was the appearance of large cell clumps near the I-cell. Associations of more than two or three cells were never encountered prior to this time and even these were purely transient. In the series shown, the I-cell itself became part of a clump as its nearest neighbors began to

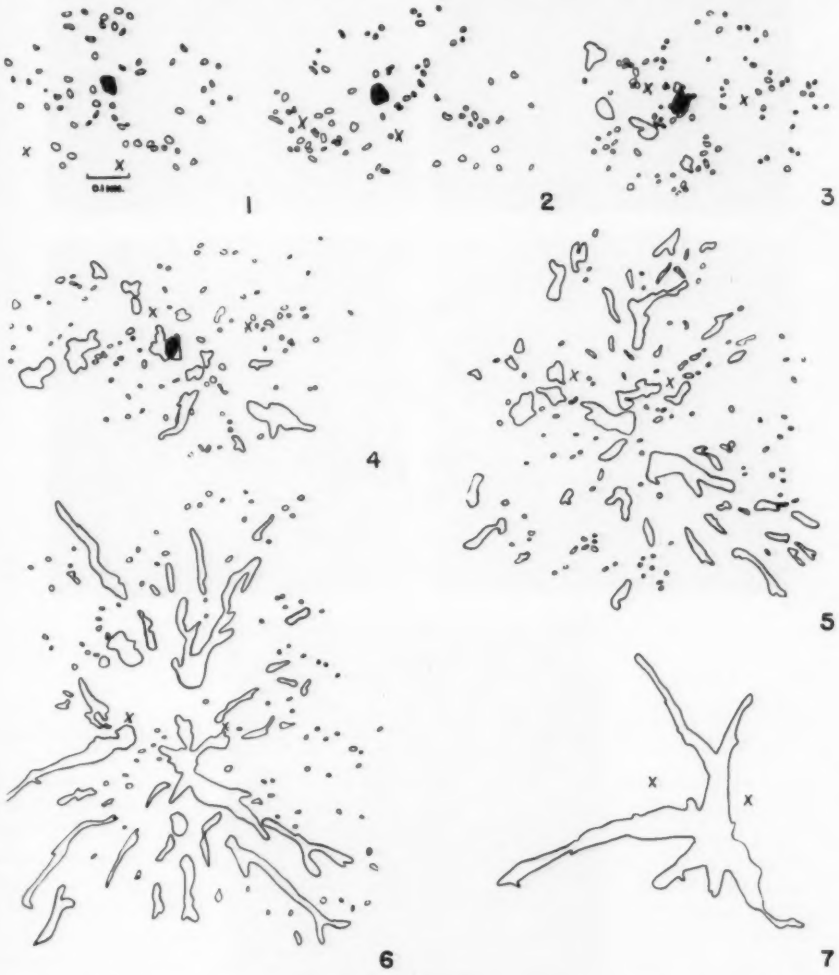

FIGURE 2. Time lapse camera lucida drawings of aggregation. The I-cell is the black individual. Cell clumps, appearing first in C, were merely outlined. The two cross-hatches mark the positions of dirt particles, used as points of reference. Respective times, in hours, after deposition on washed agar: 10.8, 11.25, 11.7, 11.9, 12.4, 13.0, 14.4.

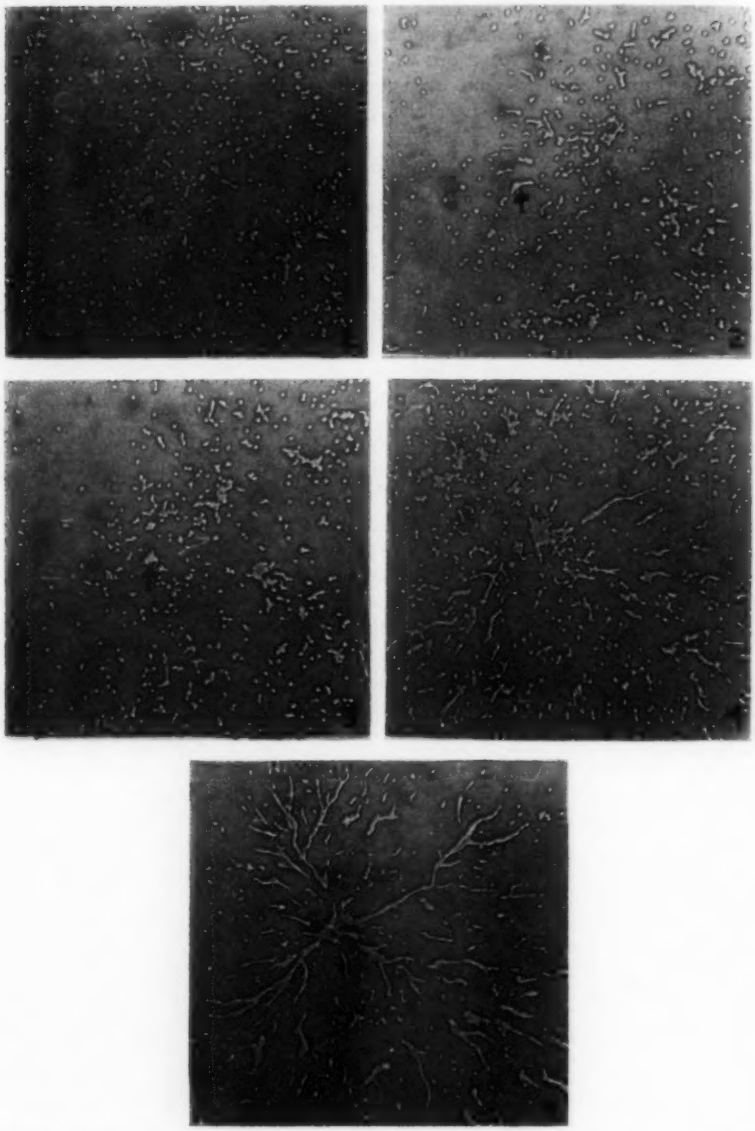

FIGURE 3. Time lapse photomicrographs of an aggregation. The arrows point to the I-cell. In photograph No. 2, the I-cell was joined by a few neighboring R-cells to form a tiny central clump. Photograph No. 3 was the last clearly discernible position of the I-cell.

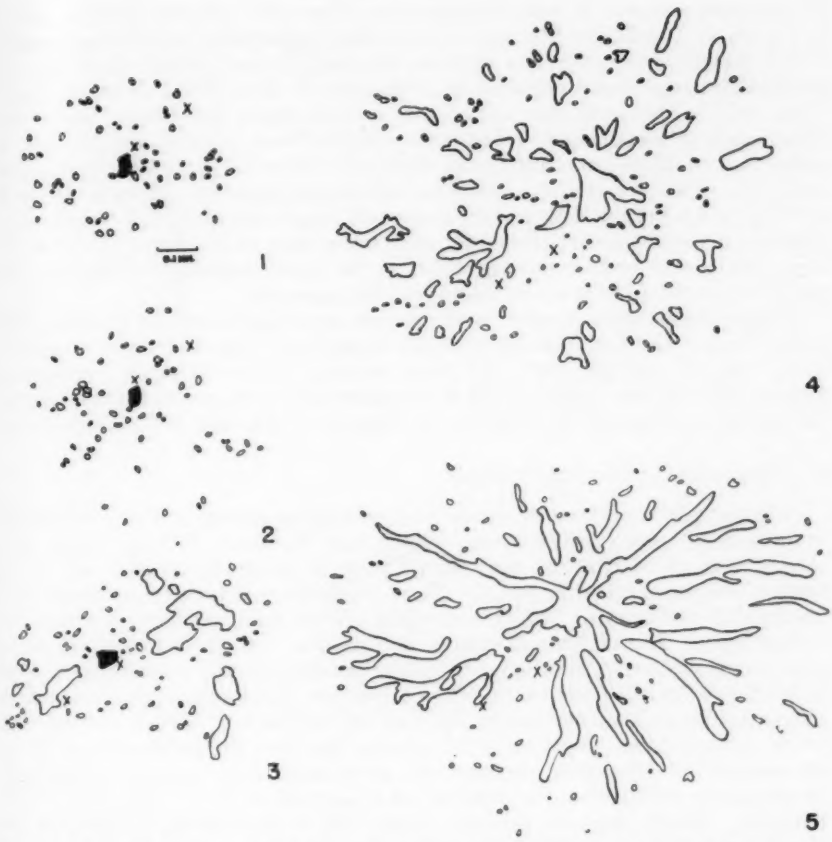

FIGURE 4. Time lapse camera lucida drawings of an aggregation. See legend of Figure 2 for details. Respective times, in hours after deposition on washed agar: 12, 12.5, 13, 13.4, 14.

nestle against it. This occurred in all but a few of the aggregations studied. The last clearly discerned position of the I-cell is in Figure 2-D. Its position was barely visible in Figure 2-E as the I-cell enlarged and extended to the right. Meanwhile, the previously formed clumps enlarged and new ones appeared concentrically about and at progressively greater distances from the I-cell. At this time, the loose cells and those in the clumps elongated and oriented radially. This caused the clumps to attain the appearance of streams. The position of the aggregative center then emerged clearly (Fig. 2-F) and is seen to have occupied the last known position of the I-cell. Ultimately the streams moved into and joined the center, producing the usual conical cell mass. Figure 3 is a series of photomicrographs of another aggregation in which the center again formed at the I-cell.

A typical sequence in which a center was established near the I-cell is shown in Figure 4. Again, the first sign of impending aggregation was the appearance of cell clumps, although on this occasion, no clump formed around the I-cell. A particularly large clump appeared at a distance of about 200 μ from the I-cell (Fig. 4-C). The I-cell then moved into a small clump immediately above the upper right reference mark. The cells elongated and oriented radially and a center was established at a distance of about 100 μ from the last known position of the I-cell. Thus the only real difference between the sequences shown in Figure 2 and Figure 4 is the appearance of the abnormally large clump, and this event always preceded the establishment of a center near, rather than at, the I-cell. In 10 of 19 cases, the R-cells entered the aggregate but the I-cell remained outside. In the other 9 cases the I-cell was also swept into the aggregate.

Figure 5 shows that a center need not form at a point along the previous path of the I-cell. The four cases were chosen because the respective I-cells wandered along relatively straight paths and could therefore clearly illustrate that such a relation did not exist. Cases 3 and 4 are particularly pertinent in that the centers did not form at the I-cells but did so at distances of 250 and 300 μ, respectively.

B. Aggregation after I-cell removal

The fact that I-cell removal can prevent subsequent aggregation was established in previously reported experiments (Ennis and Sussman, 1958b). They also showed that, to be effective, the removal must be accomplished at a very early stage of the pre-aggregative period. Thus, I-cells were removed from drops containing 500 myxamoebae within two minutes after they had been dispensed on washed agar. The incidence of centers 16 hours later (at which time all aggregations were completed), was only 9% of the incidence in the control drops from which I-cells had not been removed. If, however, the I-cells were permitted to remain for about 5 minutes before removal, the incidence of centers rose to 40% of the control value. Removal at 20 minutes increased the incidence to 67% of the controls and removal at one hour was totally ineffective, *i.e.*, equal percentages of aggregates developed in the controls and in populations from which I-cells were removed. Clearly, then, the presence of the I-cell in the immediate vicinity of the R-cells, even for a few minutes, is sufficient to produce an inductive effect.

Experiments performed since then have indicated that the I-cells can exert this inductive effect upon R-cells well outside of their immediate vicinity, albeit they require more time to do so. Replicate samples of 6000 washed myxamoebae were dispensed on washed agar at the optimal density of 200 cells/mm.^{2}. The excess fluid was absorbed by the agar and after one hour's incubation, an area, 1 mm.^{2}, was delineated at the center of each drop by scoring the agar surface lightly with two pieces of razor blade, mounted parallel at a distance of 1 mm. The cells outside of the square were brushed away, thereby leaving replicate samples of 200 myxamoebae at a density of 200 cells/mm.^{2}.

Since the distribution of I-cells has been found to be 1:1940, one would expect about 10% of the squares to have contained I-cells and accordingly to have aggregated. As may be seen in Table I, precisely 10% of the squares so treated did aggregate. Thus, it can be said that all of the aggregates observed must have been contributed by those squares that contained I-cells and that no I-cells lying

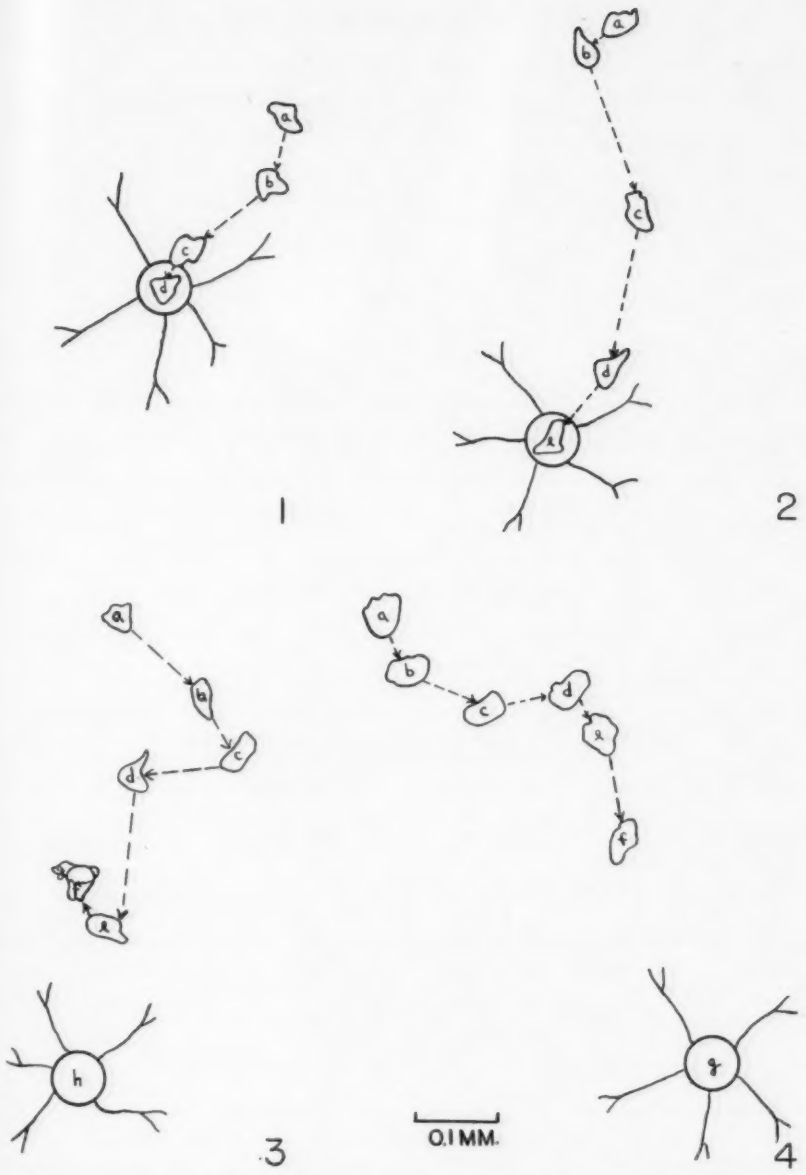

FIGURE 5. Relation between aggregative centers and previous migratory pathways of I-cells in four aggregations. In the top two, the centers coincided with the final positions of the I-cells. In the bottom two, they did not. The respective times, in hours, at which the first and last camera lucida drawings were made after deposition on washed agar: 10.8, 11.9; 9.8, 12.25; 9.8, 14.0; 10.0, 13.0.

TABLE I

After the stated periods of pre-incubation, squares were scored on the agar surface and outlying cells were brushed away. See text for details.

Pre-incubation period in hours	No. of squares	No. with aggregates	%
1	120	12	10.0
4–5	120	20	16.7
6–8	120	59	49.0
10–12	120	109	91.0

outside the boundaries of said squares for the one-hour period and then brushed away, appear to have exerted an inductive effect upon the R-cells lying within. When, however, a period of 4–5 hours elapsed before the squares were delineated and the outlying cells removed, the incidence of aggregates within the square rose to 16.7%. After 6–8 hours the incidence was 49% and after 10–12 hours, 91%. (At 12 hours, but not at 10, the cells were elongated and were beginning aggregation.) Thus, even at 4–5 hours, significantly more aggregates appeared than could be accounted for simply by the presence of I-cells within the squares. The data would therefore seem to force the conclusion that the I-cells lying outside the squares must have exerted an inductive effect upon the R-cells within. It is important to note that, since the total cell density was 200 cells/mm.^{2}, the density of the I-cells would have been about 1 per 10 mm.^{2}. Thus the I-cells in exerting their effect acted over truly fantastic distances.

The objection may be raised that not only the outlying I-cells were removed but also the outlying R-cells. Why, then, could one ascribe the inductive effect to the latter? The answer lies in the fact that when replicate samples of from 250 to 2000 myxamoebae were dispensed at densities even greater than 200 cells/mm.^{2}, a Poisson distribution of aggregates was obtained in strict accordance with the distribution of I-cells. If R-cells had any inductive capacity of their own at these densities, why then did not every sample aggregate regardless of whether an I-cell was present or not?

In summary, the data indicate that the I-cell performs its mission at the early stages of the pre-aggregative period. The immediate neighbors of the I-cell require its presence for only a few minutes and can then subsequently aggregate in its absence. The more remote neighbors can also be affected if the I-cell is allowed to remain for a longer period of time. It is difficult to explain these results save by the assumption of a diffusible "initiator substance."

C. The initiative capacity of R-cells

As mentioned previously, when I-cells were micromanipulated to test areas, they could induce the test populations to aggregate, whereas R-cells at the same stage of development could not (Ennis and Sussman, 1958b). In these experiments, the I-cells and R-cells were micromanipulated to the test areas within 20 minutes after they had been dispensed on washed agar. The question arose as to whether or not R-cells which had been incubated for periods longer than 20 minutes prior to micromanipulation might not display initiative capacity.

TABLE II

After the stated periods of pre-incubation, R-cells were individually micromanipulated to test areas. See text for details

Pre-incubation period in hours	Experimental			Background		
	Total	No. with aggregates	%	Total	No. with aggregates	%
1	53	7	13.2	79	11	13.9
4–6	65	14	21.6	250	29	11.6
10–12	71	26	36.6	70	9	12.8

Washed myxamoebae were dispensed on washed agar at a density of 150–200 cells/mm.^{2}. After 1, 4–6, and 10–12 hours, R-cells were picked up individually with a glass loop mounted in a deFonbrune micromanipulator and moved to test areas. The test areas had been prepared by dispensing washed myxamoebae on washed agar at a density of 250 cells/mm.^{2}, one hour prior to use. After the excess fluid had been absorbed, an area, 1 mm.^{2}, was delineated in the middle of each drop as described in the previous section. The outlying cells were brushed away leaving test squares containing 250 myxamoebae at a density of 250. The center:cell ratio being 1:2200, one would expect 11.3% of the squares to have aggregated spontaneously. The background controls shown in Tables II and III showed an incidence of 72 squares with aggregates out of a total of 578, or 12.4%. The extent to which addition of R-cells, pre-incubated for periods between 1 and 12 hours, affected the background incidence is shown in Table II. R-cells pre-

TABLE III

Initiative capacity of R-cells tested upon their developmental juniors

A. Samples with I-cells			Samples without I-cells		
No.	No. with aggregates	%	No.	No. with aggregates	%
21	18	86	13	0	0

B. Experiment	R-cells from samples with I-cells			R-cells from samples without I-cells			Background		
	Total	No. with aggregates	%	Total	No. with aggregates	%	Total	No. with aggregates	%
A	27	5	18.5	27	8	29.6	54	8	14.8
B	27	8	29.6	27	3	11.1	53	8	15.1
C	36	8	22.2	30	7	23.3	72	7	9.7
Total	90	21	23.4	84	18	21.4	179	23	12.8

A. Samples of 500 cells were dispensed on washed agar. Twenty-one which certainly contained I-cells and 13 which certainly did not were chosen. The percentages of samples that produced aggregates are shown.

B. After 8 hours' pre-incubation, R-cells, taken from the samples with and without I-cells, were micromanipulated to test areas. See text for details.

incubated for one hour did not affect the background frequency but increases of 10 and 24% over background were obtained by adding R-cells pre-incubated for 4–6 and 10–12 hours, respectively. In other words, when pre-incubated for 10–12 hours and then moved to test areas, one out of four R-cells could induce the formation of a center among the test cells, 12 hours after its introduction.

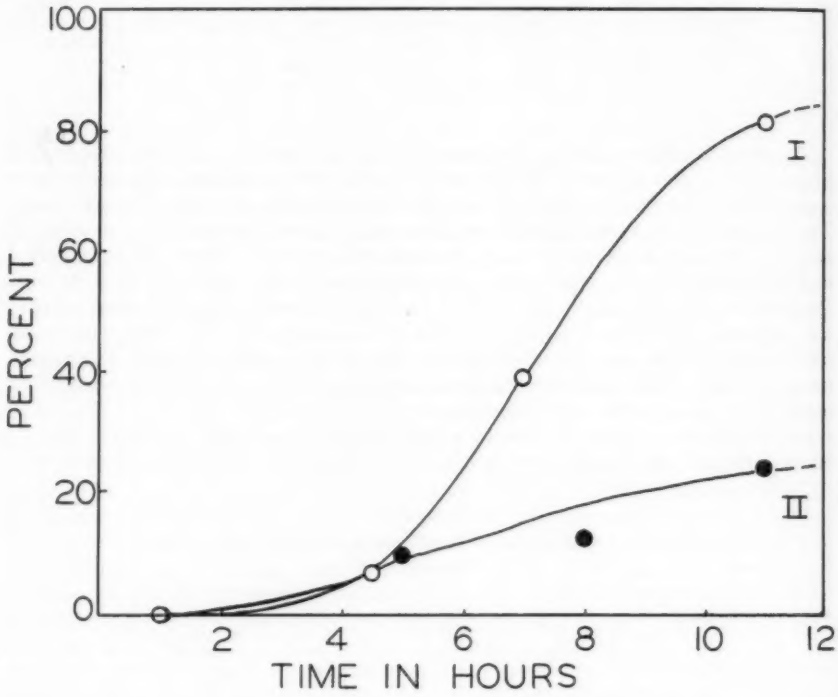

FIGURE 6. A kinetic comparison of: I. The capacity of small population samples to aggregate when isolated from their neighbors after varying periods of incubation. Ordinate: per cent of 250 cell samples that aggregated. Abscissa: time of incubation on washed agar prior to isolation. (Data from Table I.) II. The capacity of R-cells incubated for varying times on washed agar to initiate centers amongst their developmental juniors. Ordinate: per cent of R-cells capable of initiation. Abscissa time of incubation on washed agar prior to their micromanipulation to test areas. (Data from Table II.)

Figure 6 is a graphic comparison of the kinetics of induction of centers in test squares (I) by progressively delayed removal of outlying I-cells (data from Table I) and (II) by addition of pre-incubated R-cells (data from Table II). The crude kinetic similarity suggested that the outlying I-cell might not only be responsible for the subsequent aggregation of the R-cells but also for the concomitant increase in their capacity to themselves initiate centers. To test this possibility, replicate samples

of 500 washed myxamoebae were dispensed on washed agar. In three experiments 21 samples were chosen which certainly contained I-cells and 13 which certainly did not. The data in Table III confirm the correctness of these choices since 86% of the samples said to contain I-cells aggregated while none of those said not to contain I-cells did so. After these samples had been incubated for 8 hours, R-cells were picked and moved to test squares as described in the preceding paragraph. Table III shows that R-cells, whether pre-incubated in the presence or absence of I-cells, were equally capable of inducing center formation. Thus, the rise of the initiative capacity of the R-cells during the pre-aggregative period is not dependent upon their contiguity with I-cells. Two points must be kept in mind here. First, it must be remembered that prior to their deposition on the washed agar, R-cells had all been in contact with I-cells and therefore could have been at this time the subject of interactions emanating from the latter. Second, even though the R-cells after 12 hours of incubation had attained a significant degree of initiative capacity, they fell far short of the level displayed by the I-cells after only 20 minutes of incubation. Therefore, the phenotypic difference between the two cell types in this respect remains clear.

Finally, the results reveal a most puzzling paradox. When R-cells were pre-incubated for 8 hours in the absence of an I-cell and then placed in the presence of test cells for an additional 12 hours, at least one out of ten could induce center formation. Yet the samples from which these R-cells originally came, when incubated for a total of 20 or indeed 36 hours, had not aggregated. It is clear, therefore, that the observed increase in the initiative capacity of R-cells during the pre-aggregative period in the development of a population is of no consequence to the ultimate aggregation of *that* population. In other words, the initiative capacity of such R-cells, demonstrated by movement to another population, is an experimental artifact bearing no relation to normal aggregation but which may possibly be used to understand the biochemical and genetic differences between the I-cell and R-cell *Phenotypes*.

Discussion

The data presented here and previously suggest a developmental program of slime mold aggregation that may serve as a useful working hypothesis.

I-cells arise during the growth of an R-cell population (which in turn had originated from the spores of the preceding fruit), and attain a steady-state ratio of approximately 1:2000 early in the exponential phase (Sussman, 1956; unpublished data). Entrance into the stationary phase marks the beginning of the pre-aggregative period. At the beginning of this period, the I-cells secrete material which, during the ensuing 12 hours, so conditions the neighboring R-cells as to induce them to aggregate. This interaction, as might be expected, affects the nearest neighbors first but its influence is progressively extended. Concomitant with, but unrelated to either the presence of the I-cell or the subsequent course of aggregation in the same population is a significant rise in the initiative capacity of the R-cells themselves. Such cells upon extended incubation never do attain the degree of initiative capacity displayed by the I-cells nor can they act upon their developmental contemporaries but only upon cells at an earlier developmental stage to which they have been added by the observer.

The first overt sign of aggregation is the formation of cell clumps concentrically about and usually at the I-cell. This is followed by excitation and elongation of the loose and clumped cells in response to the chemotactic complex (Sussman *et al.*, 1956; Shaffer, 1956; Sussman, 1958). The appearance of oriented streams establishes the position of the aggregative center. This is usually coincident with the final position of the I-cell but sometimes with the position of a particularly large clump nearby, and possibly reflects the point of greatest production of the chemotactic complex. In the latter case, the position of the center need bear no relation to the previous path of the I-cell.

The picture as drawn raises many questions and offers a number of predictions under current study. The most important of the latter involves the hypothetical existence of an "initiator" substance. In view of the I-cell removal experiments, one ought under the same conditions to be able to induce test cells to aggregate by dispensing them in an area previously but no longer occupied by an I-cell. This is being tested. The I-cell addition experiments raise the question as to what is the minimum period of time after contact with the I-cell in which the induced R-cells can begin aggregation. Is the 12-hour period subsequent to contact mandatory or does it involve preparations by the R-cells for aggregation, unconnected with the function of the I-cell? In the latter case, one ought to be able to preincubate the test cells for twelve hours, add I-cells, and observe the onset of aggregation very shortly thereafter.

The fact that R-cells can also attain initiative capacity to a far smaller degree, albeit much later than do the I-cells and ineffectively so far as inducing their contemporaries to aggregate is concerned, still suggests that the metabolic pathways involved in initiation are not unique to the I-cells. Indeed, one may imagine that the sole basis for the difference between I-cells and R-cells in this respect is the much greater size of the former. Perhaps, then, any of the diverse methods for producing giant cells may serve to create initiators just as does the normally occurring R-cell to I-cell transformation. This point is also under current study.

Summary

Dictyostelium discoideum myxamoebae occur as two distinct morphological types, termed I-cells and R-cells. Data presented in a previous publication demonstrate that I-cells can initiate centers of aggregation and suggest compellingly that they are in fact the initiator cells for normal aggregation. The present communication extends and amplifies these findings.

A. Time lapse camera lucida drawings and photomicrographs illustrate the sequence of events during the onset of aggregation.

B. Small population samples of myxamoebae, when isolated from their neighbors shortly after deposition on washed agar, showed a distribution of aggregative centers consistent with the distribution of I-cells within the samples. Longer periods of contact with neighboring cells (including other I-cells) that surrounded the samples prior to isolation permitted progressively greater proportions of the samples to aggregate. The possibility arises of an "initiator substance" whose effect may extend over relatively great distances.

C. R-cells, incubated for long periods of time on washed agar, were found to have acquired initiative capacity. At best, only a small proportion did so and fur-

thermore could only induce the formation of aggregative centers amongst their developmental juniors (by twelve hours) but not amongst their developmental contemporaries.

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SHELL REPAIR IN CHITONS

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Cryptochiton stelleri (the "gumboot") is not only the largest member of the class Amphineura, but also one of the most specialized in that the girdle tissue has completely overgrown the skeletal plates (Heath, 1897). It therefore lacks the outer shell layer, the tegmentum. While preparing some of the skeletal plates for display, it was noticed that occasional plates were cracked and that many of these cracks were repaired by an amber-colored membrane resembling conchiolin. It seemed of interest to determine the frequency of damage, the stages of repair, the possible significance of this ability to the survival of the animal, and the relative incidence of breakage and repair in several other species of chitons (*Katherina tunicata, Mopalia hindsii*).

Cryptochiton (*Amicula*) *stelleri* is a subtidal browsing herbivore, but it is also found in fair numbers up into the middle zone of the intertidal region. When found in the intertidal zone it is attached loosely to rock encrusted with coralline algae or to algal curtains, and occasionally it is found on a sandy bottom. *Cryptochiton* holds to its substrate only gently and can be removed easily by hand. It is also dislodged by wave action as evidenced by the large number (approximately 75) counted on three local beaches after a heavy storm in April, 1958. The plates of the storm-tossed animals were shattered and all but five of the animals were dead. It is possible that after seeking food in shallower tidepools and crevices during high water, the chiton is left by the subsequent receding tide and falls from its loosely-held position among the algae. Caught by wave action, it may be beaten against the rocks before it can re-establish its hold or before it can get back to deeper waters. The animals when strongly stimulated in the laboratory have been seen to contract with sufficient force to crack their plates; perhaps some are also broken in this manner in nature.

Of the 146 sets of plates (Fig. 1A) collected [2] 87, or 59.5 per cent, had one or more plates broken (about 18 per cent had one, 17 per cent had two, 11 per cent had three, 6.2 per cent had four, 3.4 per cent had five, 2.7 per cent had six, 1.3 per cent had seven, but none had all eight). Two animals had seven of the eight plates broken. The middle plates were broken most often, these being the widest and flattest (6.2 per cent plate 1, 11.4 per cent plate 2, 14.1 per cent plate 3, 18 per cent plate 4, 21.2 per cent plate 5, 16.8 per cent plate 6, 8.4 per cent plate 7, and 3.9 per cent plate 8). Often two or three adjacent plates were found with similar breaks, suggesting a blow from a large surface.

[1] Supported in part by U. S. Public Health Grant RG 4578 to A. C. Giese.

[2] The chitons were being used in a study of the annual reproductive cycle and of the biochemistry of the blood and tissues; hence the plates were available in numbers, from specimens collected for these purposes.

Plates that had been broken just prior to the animal's death, either by storms or in the laboratory, showed a clean cleavage with the parts fitting perfectly together. Depending upon the severity of the blow, the cleavage was in a single straight line or in an arborescent pattern. Repairs were seen in few plates that had been shattered into as many as seven pieces.

The first step in repair is the formation of a strip of membrane overlapping the crack on both sides of the plate. The second stage seems to be the accumulation of fine granules of a calcium salt, presumably in the form of carbonate (Bevelander and Benzer, 1948), under the conchiolin strip with the concomitant erosion of the underlying crystalline shell. This erosion often extends for some distance laterally from the crack under an extension of the membrane strip (Fig. 1C). The last step in repair (Figs. 1D–G) is the invasion or growth of existing minute crystals (Bevelander and Benzer, 1948; Bevelander, 1953) of the surface of the membrane strip by crystals of calcium carbonate in the form of aragonite (Prenant, 1927). The crystals are imbedded in the surface of the conchiolin, leaving a ridge over the crack, and often an air space or a layer of membrane between the old shell and the new material (Fig. 1E). This leaves the plate weakened so that a second blow usually splits the plate along the old crack.

To determine the rate of repair of broken plates, five chitons subjected to hammer blows were kept in the laboratory with ample food and in running sea water, and sacrificed after varying lengths of time. The results are quite variable but they serve to illustrate the slowness of repair. For example, while one chiton developed membranes around the cracks in twenty days and granular calcium carbonate deposition in twenty-four, another showed no visible sign of repair in the same period of time. In still another chiton, dissected sixty days after breaking the plates, crystalline calcium carbonate was evident in the cracks. However, in two chitons examined 100 days after injury, only membranes had been laid down over the cracked edges of the plates.

Energy for mobilization of the shell calcium is available only during active feeding and digestion in some mollusks (Wagge, 1951, 1952; Robertson, 1941). The effects of starvation were not tested here in view of the variability of results with well-fed specimens.

Wilbur and Jodrey (1955) inhibited shell deposition in the oyster with carbonic anhydrase inhibitor. However, no tests were made with such inhibitors on *Cryptochiton* in view of the variability of results and the long time required for repair of broken skeletal plates. Furthermore, it is not even known whether amphineurans possess carbonic anhydrase although Freeman and Wilbur (1948) found it in most, but not all, of the species of gastropods and pelecypods tested.

Katherina tunicata

Fifty-five sets of plates of *Katherina* were examined and only five plates were found to be broken, although many of them were eroded to some extent, possibly by a disease. Of these, two showed slight evidence of repair. One had a thin membrane with a few lime crystals, the other showed an old crack completely repaired. It is possible that the other cracked shells were broken when the eviscerated specimens were boiled to loosen the plates for examination. One valve broken experimentally showed a conchiolin membrane after a few weeks.

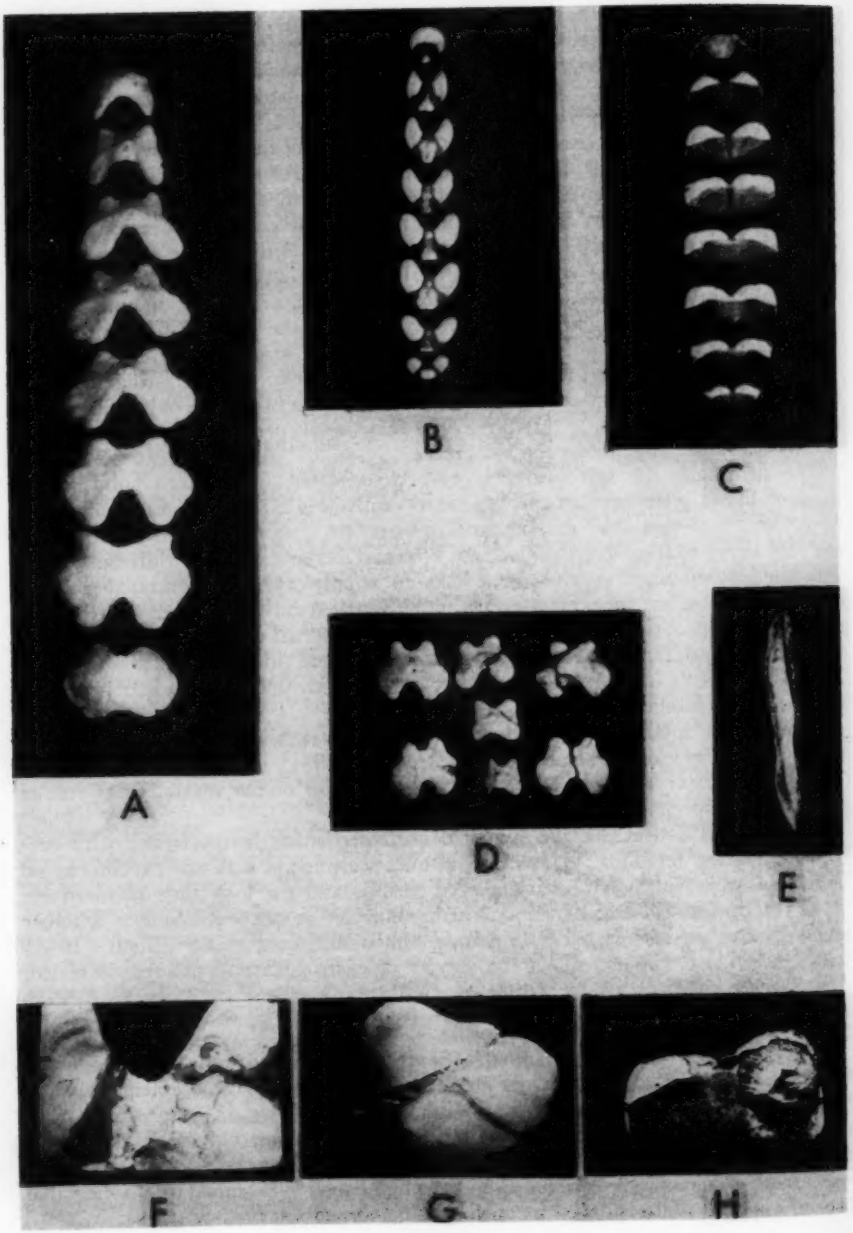

FIGURE 1.

Katherina lives in the surf zone on exposed shores among the sea palms and between mussel beds where, at certain times of the day, it withstands an almost continual pounding by the waves. Even at low tides the animals hold fast to bare rock or crustose algae with such strength that a knife or screwdriver is needed to pry them loose. At that, an inexperienced collector will often get only the plates and girdle, the foot and viscera remaining on the substrate. The storms of 1958 which left so many specimens of *Cryptochiton* on the beaches presumably failed to dislodge specimens of *Katherina;* at least none was seen on the beaches with *Cryptochiton.*

The infrequency of broken plates in *Katherina* suggests that its plates are proportionally stronger than those of *Cryptochiton.* The average weight of the eight plates (10 specimens; average wet weight 33 grams) was 19.6 per cent of the wet weight of the entire chiton, in comparison to the 7.4 per cent for *Cryptochiton* (45 specimens; average wet weight 850 grams).

It is also possible that the shape of a skeletal plate has some bearing on its resistance to shock. Plates No. 2 to No. 7 of *Cryptochiton* are in the shape of butterflies (Fig. 1A) and are relatively flat. The skeletal plates in *Katherina* consist of a heavy, roughly circular, disc with one pair of thin lateral lobes (Fig. 1B).

Mopalia hindsii

Twenty-six sets of skeletal plates of *Mopalia* were examined and, other than chipping along the edges of the thin membrane, eleven had broken plates (six had one plate broken, three had two and one each had three or four plates broken). The most common crack was from the lateral notch to the beak of the plate. Along this line the plate is porous. Although many of the cracks are clean and may have resulted from boiling eviscerated specimens to release the plates, *Mopalia* suffers a fairly high incidence of infection from an unidentified boring animal which weakens the prismatic layer of the plate with long tunnels. One of these weakened plates was broken and the shell was thickened along the cracked tunnel. A thin membrane and some lime crystals were also deposited after a lapse of several weeks along cracks in plates No. 2 to No. 7 broken by a blow from a hammer. One unbroken plate showed deposition of new material where an attached barnacle overlapped the edge of the plate (Fig. 1H). *Mopalia* therefore can to some extent repair its skeletal plates.

The specimens of *Mopalia* used in this work were collected from concrete pilings in Monterey Harbor, a relatively protected habitat. While the skeletal plates are broad and flat (Fig. 1C) and in this respect resemble those of *Cryptochiton,* at the same time they constitute about 22.1 per cent of the wet weight of the animals. Apparently they are adequate for the conditions to which the animals are exposed.

FIGURE 1. A. Shell plates of *Cryptochiton stelleri.* × ½. Plate No. 1 (anterior) is toward the top of the page. B. Shell plates of *Katherina tunicata.* × ½. Plate No. 1 (anterior) is toward the top of the page. C. Shell plates of *Mopalia hindsii.* × ⅔. Plate No. 1 (anterior) is toward the top of the page. D. Representative cracks in shell plates of *Cryptochiton* undergoing repair. × 1/10. E. A section along a crack in a plate of *Cryptochiton* showing the space between the old shell and the new material deposited during repair. × 1. F. Lateral extension of the conchiolin strip (dark material) over two breaks. × 1. G. Invasion of the conchiolin strip (dark) by crystalline calcium carbonate (light). × 1. H. Deposition of new shell (above, right) along edge of barnacle attached to *Mopalia plate.* × 1.

Summary and Conclusions

The skeletal plates of *Katherina tunicata* and *Mopalia hindsii* are sturdy, constituting about a fifth of the wet weight of the animal. They were seldom found broken in the specimens examined, but some broken plates were undergoing repair. The skeletal plates of *Cryptochiton stelleri*, on the other hand, are flat and thin and constitute only 7.4 per cent of the wet weight of the animal. The majority of cryptochitons examined showed breaks in one or more skeletal plates and in almost all of these, some degree of repair and deposition of membrane or mineral could be observed. The ability to repair its plates is probably of value to this species in view of the weakness in design of its skeleton.

Irregularities of plates and variations in numbers of skeletal plates have been described for other species of chitons (Crozier, 1919; Berry, 1925, 1935; Taki, 1932). It is interesting that apart from an occasional asymmetrical terminal plate of a *Cryptochiton*, no such irregularities in number or shape were observed in the three species of chiton studied here.

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THE JUVENILE HORMONE. I. ENDOCRINE ACTIVITY OF THE CORPORA ALLATA OF THE ADULT CECROPIA SILKWORM

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The endocrine role of the corpora allata of insects was discovered by V. B. Wigglesworth (1934, 1936) over twenty years ago. In a series of simple and decisive experiments on *Rhodnius* he showed that the corpora allata secrete a "juvenile hormone" which opposes metamorphosis. In these early studies Wigglesworth also recognized that the corpora allata undergo pronounced changes in endocrine activity during the course of metamorphosis; namely, that they are active in the immature nymph, inactive in the mature nymph just prior to metamorphosis, and active again in the adult insect after metamorphosis. Subsequently, the general validity of these conclusions has been confirmed repeatedly and found to apply to both hemi- and holometabolous insects (for review, see Wigglesworth, 1954, pages 56–64).

During the past twelve years, in the course of studies of the metamorphosis of the Cecropia silkworm, the juvenile hormone has necessarily been an object of detailed attention. While confirming the essential elements in Wigglesworth's theory, the study has helped to resolve certain persistent mysteries and, more recently, has pointed the way to the successful extraction and purification of the hormone itself. This first of a series of communications is concerned with the endocrine activity of the corpora allata of the adult moth.

Materials and Methods

1. *Experimental animals*

The experiments were performed on Cecropia, Cynthia, and Polyphemus silkworms. Taxonomists continue to amuse themselves by changing the generic and specific names of these Saturniids. What began as *Phalaena cecropia* became *Samia cecropia*, then *Platysamia cecropia*, and now *Hyalophora cecropia* (Michener, 1952). The Cynthia silkworm, known throughout the world as *Philosamia cynthia*, was changed to *Samia walkeri*, and then back to *Samia cynthia*. *Telea polyphemus* is now *Antheraea polyphemus*. As in the analogous cases discussed by Wald (1952, page 339), the "common names" have escaped the attention of taxonomists and have remained firm and unchanging. Therefore, the common names will be used routinely in the present reports.

[1] This study was aided by a grant from the National Institutes of Health of the U. S. Public Health Service. It is a pleasure to acknowledge the advice and counsel of Prof. Berta Scharrer.

Cecropia silkworms were reared under nylon nets on wild-cherry trees. Polyphemus were reared on oak or maple; Cynthia, on cherry or ailanthus or purchased from dealers. The cocoons were harvested and stored as previously described (Williams, 1946a; Shappirio and Williams, 1957).

2. *Surgical procedures*

Experimental animals must be deeply anesthetized during surgical procedures. We use carbon dioxide for this purpose and with mixtures of air and carbon dioxide have maintained pupae anesthetized for as long as one month without injury. Groups of animals are placed in a capped, flat-bottom Büchner funnel and exposed for about twenty minutes to a slow stream of carbon dioxide from a compressed cylinder. The gas is bubbled through water *en route* to the funnel. The animals are flaccid when fully anesthetized, and one can no longer elicit any movements of the abdominal segments.

Surgical procedures are performed in a second Büchner funnel (diameter 11 cm., height 3 cm.) which is mounted flush on the top of the operating bench. A slow stream of carbon dioxide is bubbled through water and passed through the bottom of the uncovered funnel. Carbon dioxide, being heavier than air, fills the cavity of the funnel and maintains a continuous anesthesia during the surgical procedure (Williams, 1946b).

Operations are carried out under the low magnification of the dissecting microscope, making use of 9 × oculars and 0.7, 1, or 2 × objectives. The foot of the microscope is removed and the vertical pillar permanently attached to the operating bench on the distal side of the funnel. A hinged-arm permits the microscope to scan the entire diameter of the funnel. In order to leave both hands free, the microscope is equipped with a foot-focusing device (designed and built by Mr. Robert Chapman of the Harvard Biological Laboratories). Illumination is provided by a 6-volt microscope lamp (Zeiss "Osram") attached to and moving with the microscope. The lamp is equipped with an infra-red filter.

Anesthetized animals are transferred to the carbon dioxide-filled funnel for the surgical procedure. They are then returned to air, placed in individual numbered glass containers ("creamers"), and stored in a room having a controlled humidity of sixty per cent and a temperature of 25° C.

Dissecting instruments consist of the following: watchmaker's forceps (Dumont "rustless"; two of No. 3 and two of No. 5); a scalpel (Bard-Parker No. 3 handle with a No. 11 detachable blade); stainless iris scissors curved on the flat and closing to the tip; several forms of stainless steel iridectomy and micro-scissors; a stainless steel dental probe; a 5-ml. hypodermic syringe filled with insect Ringer and capped with a 25-gauge needle.

Prior to each group of operations the instruments are briefly rinsed in seventy per cent ethanol and wiped dry. Rigorous asepsis is unnecessary because the blood of the silkworms apparently contains an anti-bacterial substance that protects it from the ordinary contaminants. However, it fails to protect from insect pathogens and no diseased insect should be operated upon with the same instruments or even in the same room.

Healthy pupae can withstand almost any degree of surgery provided that a few crystals of the potent anti-tyrosinase, phenylthiourea, are placed in the operat-

ing field. We routinely use an equal part mixture of phenylthiourea (twice recrystallized from hot 95 per cent ethanol) and streptomycin sulphate, the two having been ground together in a mortar and stored in a capped vial in the refrigerator. Small amounts of the powder are removed and discarded within two days after being placed at room temperature.

Ephrussi-Beadle Ringer's solution is utilized containing 7.5 gm. NaCl, 0.35 gm. KCl, and 0.21 gm. $CaCl_2$, per liter of distilled water. The stock solution is brought to a boil, capped, and stored in the dark under refrigeration. Fungal contamination of physiological solutions, especially those containing bicarbonate, is a common source of difficulty when solutions are stored at room temperature.

Excised tissues and organs are transferred to small depression dishes made of black glass and filled with Ringer. Black plastic bottle-caps are also satisfactory for this purpose. Dissections of sacrificed animals are performed in a glass Petri dish which fits snugly into the cavity of the Büchner funnel. Plasticine is pressed into the bottom of the dish to receive short stainless steel pins. The dish is filled with Ringer and the dissection performed with the animal spread and pinned under the solution.

After surgical procedures on surviving pupae, Ringer's solution is added from a hypodermic syringe so that the blood is flush with the surface of the cuticle. The area of excised cuticle is then capped by a plastic window of appropriate size. The latter is punched or cut with scissors from cellulose acetate cover slips ("Turtox," thickness 1 or 2). The window is sealed in place with paraffin wax which is melted in an alcohol lamp and transferred with a curved needle or drawing pen. The melted wax adheres to the cuticle and the underside of the rim of the plastic slip provided that both are dry. The operating field is thereby equipped with a transparent window which permits one to look inside the living animal.

3. *Excision of pupal corpora allata and corpora cardiaca*

An anesthetized pupa is placed in a plasticine cradle in the bottom of the carbon dioxide-filled funnel. The cuticle of the facial region is first removed. For this purpose a scalpel incision is made through the integument on each side of the face. The two cuts are joined by a transverse cut and the rectangle of cuticle is grasped with forceps and pulled free from its attachment at the base of the legs. The insect's abdomen is then pressed forward with plasticine and held in this position so that the blood fills, but does not overflow, the operating field. The naked epidermis is grasped with forceps, split down the middle, and trimmed free with scissors. The brain is thereby exposed. This is pressed down in the field to reveal the tiny corpus allatum-corpus cardiacum complex on each side. The complexes are dorso-lateral to the brain and attached on each side to a large tracheal trunk at this position (see Figure 1). A pair of tiny nerves emerges from the posterior face of each brain hemisphere and passes to the corpus cardiacum on that side. These nerves are very delicate and difficult to see in a dissection of this type.

By means of forceps the connections between glandular complex and the adjacent trachea are broken, and the complex transferred to Ringer's solution in a black dish. Alternatively, the tracheal segment can be excised with iridectomy scissors and removed along with the glandular complex.

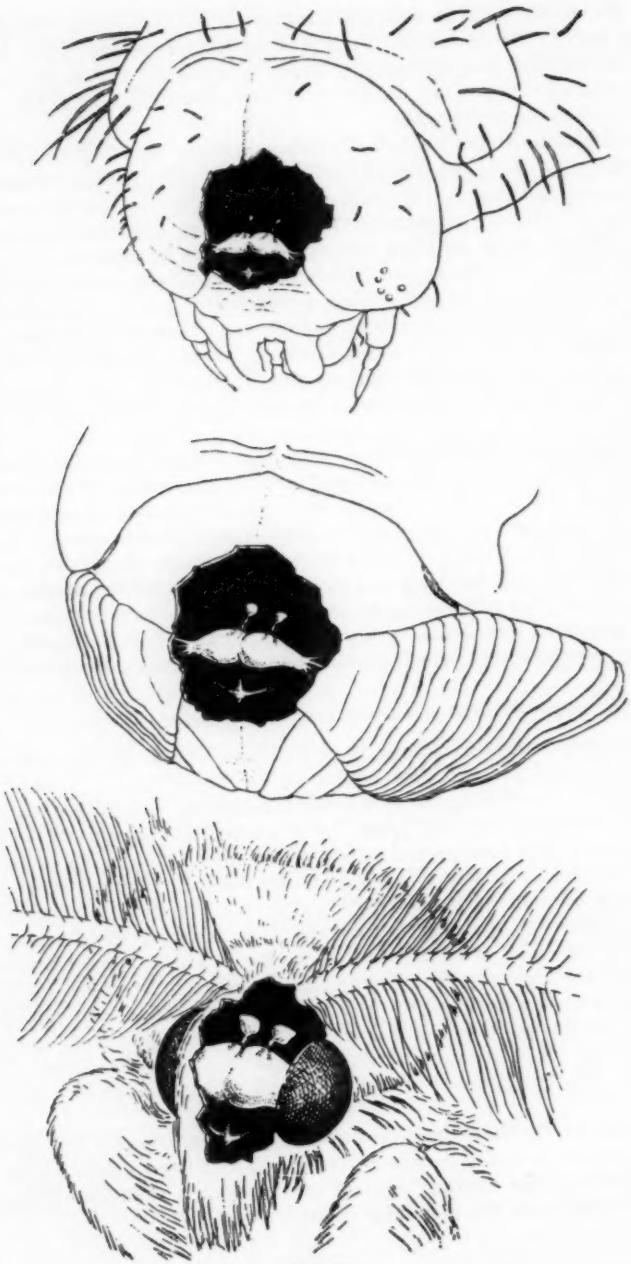

FIGURE 1.

4. *Excision of adult corpora allata*

The moth is anesthetized and its head dipped momentarily into seventy per cent ethanol to wet the scales and hairs. The head is then cut off with scissors and placed in Ringer's solution. (The headless moth will continue to live for approximately the normal life-span of 7 to 10 days at 25° C.)

The antennae are excised at their bases. Then with fine scissors the head is cut along the dorsal midline from its posterior margin to the mouth parts. The head is then spread apart with forceps and pinned under Ringer. The pair of corpora allata-corpora cardiaca complexes is attached to the aorta just behind the brain. The brain is split in the midline to expose the aorta. The glandular complexes can now be broken free from the rear of the brain and transferred to a black dish by grasping the aorta with forceps.

Under the favorable conditions of illumination in the black dish, one can recognize the corpus cardiacum; it is attached by short nerves to the much larger corpus allatum. The latter is ordinarily flattened or wedge-shaped and subdivided into a number of lobes and lobules. If necessary, the glandular complex may now be subdivided into its constituent parts by breaking the nerves between corpus cardiacum and corpus allatum.

5. *Isolation of pupal abdomens*

This procedure has already been described for the Cecropia silkworm (Williams, 1947). The principal difficulty is to isolate the terminal abdominal segments without puncturing the fluid-filled midgut. This difficulty is circumvented by the use of the Cynthia silkworm. In this species the midgut contains only a solid, rod-like mass. Therefore the perforation of the midgut is inconsequential. The pupa is transected just behind the metathorax with a single transverse cut of a sharp razor blade. The abdomen is then supported with the cut surface facing upward. Crystals of the phenylthiourea-streptomycin mixture are spread in the wound, and Ringer's solution is added to fill the cavity of the abdomen. The wound is then capped with a plastic slip in which a central hole has been punched. The plastic is sealed in place with melted wax. Ringer is finally added *via* the central hole to replace all air, and the hole itself sealed with wax.

Results

1. *Role of the corpora allata in adult development and sexual maturation*

The pair of corpora allata-corpora cardiaca complexes was removed from each of a series of twenty chilled male or female Cecropia pupae *via* the facial approach. The integumentary defect was capped and sealed with a plastic window, and the animals placed at 25° C.

Adult development was initiated after about two weeks and proceeded in synchrony with the time-table for the normal development of Cecropia at 25° C. (Schneiderman and Williams, 1954). The moths, emerging after three weeks

FIGURE 1. Brain and corpora allata of the Cecropia silkworm are shown in cutaway views of the head of larva (*top*), pupa (*middle*), and adult (*bottom*). The corpora allata are the two small bodies attached by tiny nerves to the back of the brain. (This figure is used with the permission of *Scientific American*.)

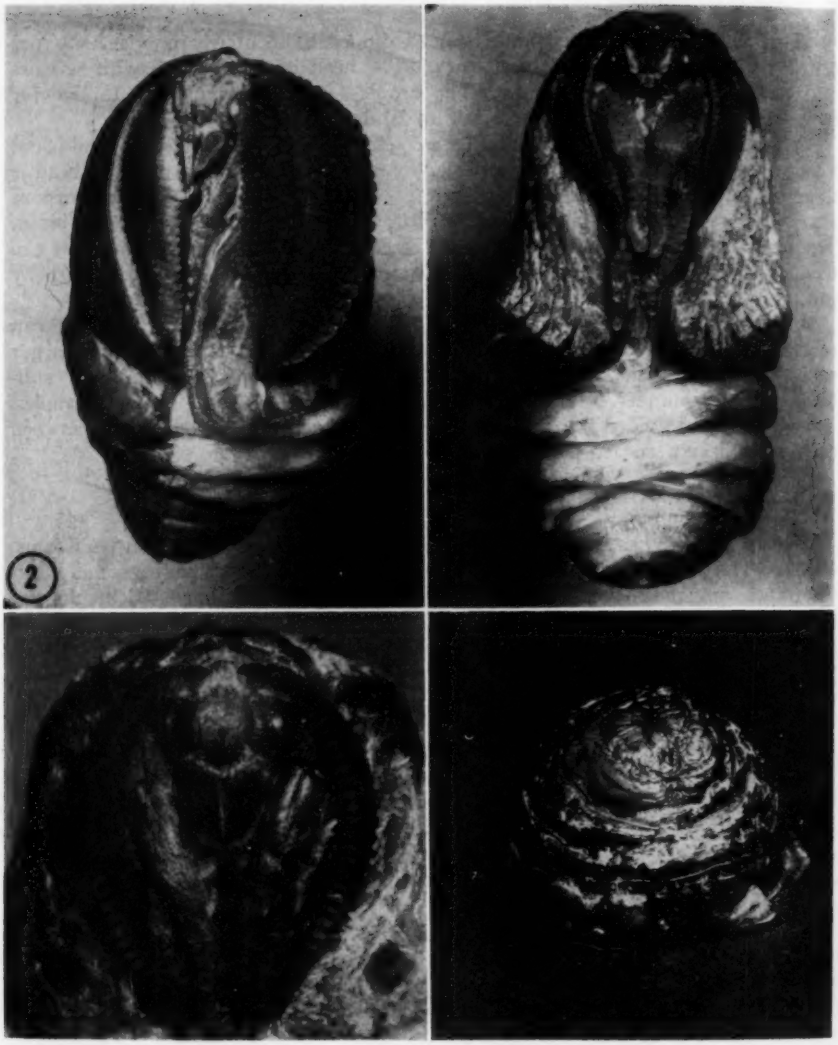

FIGURE 2. After receiving implants of three pairs of corpora allata of adult Cecropia, the Polyphemus pupa, here illustrated, has transformed into a second pupal stage. (See right side of preparation where the old pupal cuticle has been trimmed away.)

FIGURE 3. This Cecropia pupa received implants of two pairs of adult Cecropia corpora allata. Development has given rise to a mixture of pupa and adult. (The old pupal cuticle has been completely removed.)

of adult development, could not be distinguished from un-operated individuals. The females deposited a normal complement of eggs and both sexes survived for the customary period of 7 to 10 days at 25° C.

The absence of corpora allata was confirmed in dissections of many of these moths. All the internal organs, including the gonads, showed full and complete development. The abdomens of females were packed with ripe eggs, and the males showed normal spermatogenesis.

The experiment was repeated on a series of six male and six female pupae to produce moths lacking corpora allata. The two sexes were cross-mated and each of the six females was allowed to oviposit in a paper bag. A normal number (150–225) of eggs was collected from each female. These were placed under large nylon nets and the larvae reared to maturity on wild-cherry leaves. No deviation from normal development could be detected.

These experiments show that the corpora allata play no evident role in the transformation of the pupa into an adult Cecropia or in the gonadal function of the adult itself.

2. *Endocrine activity of adult corpora allata*

In the absence of any obvious function of the corpora allata of adult Cecropia, it is paradoxical to find that the glands, when excised and tested for endocrine activity, are more active in the moth than at any other stage in the life history (Williams, unpublished data). This fact was discovered eleven years ago in the course of an experiment performed for other purposes. It happened by chance that a pair of adult corpora allata was implanted into a brainless diapausing Cecropia pupa. Ten days later, the host showed the termination of diapause and the initiation of development. This result would have been puzzling in a normal diapausing pupa; in a brainless diapausing pupa it was incomprehensible.

Even more puzzling was the character of the development which then took place. Within two weeks the brainless pupa transformed, not into a moth, but into a bizarre creature in which large areas of pupal cuticle had been freshly formed (see Figs. 3 and 4). The animal, in short, was a mosaic of pupal and adult characteristics (Williams, 1952b).

During the past eleven years this result has been duplicated on numerous occasions. The experimental series includes fifty-one brainless Cecropia pupae which received one to three pairs of corpora allata-corpora cardiaca complexes derived from male or female Cecropia moths. As shown in Table I a total of twelve individuals (23 per cent) showed the result just described. The residual 77 per cent showed no effect of the implantation and continued to diapause. But the twelve positive experiments were of sufficient interest in themselves. Not only

FIGURE 4. Pupal-adult monstrosity after implantation of adult corpora allata. Note the pupal cuticle on head, palps, and antennae. However, the wings show scale-covered adult cuticle and the eyes show considerable adult development.

FIGURE 5. This isolated pupal abdomen received implants of adult corpora allata, plus an injection of ecdysone. The tip of the old pupal cuticle has been torn away to reveal a second pupal abdomen that has formed.

TABLE I

Tests of adult corpora allata-corpora cardiaca complexes in brainless diapausing pupae*

Adult donors	Brainless hosts	Normal development	Mixed development	No development
Cecropia	Cecropia	0	12	39
Cecropia	Cynthia	0	2	2
Cecropia	Polyphemus	0	2	1
Cynthia	Cynthia	0	0	5
Cynthia	Cecropia	0	0	20
Polyphemus	Polyphemus	0	2	0
Polyphemus	Cecropia	0	4	10
Totals		0	22	77

* One to three pairs of complexes from male or female moths were implanted into each brainless pupa.

had the implants caused the formation of mixtures of pupa and adult; seemingly, they also had substituted for the brain and provoked the termination of diapause.

As shown in Table I, this result was duplicated when corpora allata of adult male or female Cecropia were implanted into brainless diapausing pupae of Cynthia or Polyphemus. Here again, a certain percentage of animals terminated diapause and developed into pupal-adult mixtures.

The corpora allata-corpora cardiaca complexes of male and female Cynthia and Polyphemus moths were also tested. The three species seem to differ among themselves in the endocrine activity of the adult corpora allata. For example, the corpora allata of adult Cynthia gave negative tests in all twenty-five preparations. By contrast, the glands of adult Polyphemus gave positive tests in six of sixteen preparations. Moreover, when used as recipients of implants, brainless Polyphemus pupae seemed to have a lower developmental threshold than the other two species, for four of five individuals gave a positive reaction to the implantation of adult corpora allata. In retrospect, Polyphemus appears to be the animal of choice for experiments of this type.

In the far more numerous tests of Cecropia corpora allata, the conditions of the experiment were subjected to minor variations in the hope of recruiting a positive response in a larger proportion of individuals. By increasing the number of implanted glands from one to two or three pairs, little additional effect was realized. However, the developmental response was markedly enhanced when the host animals were placed at 15 or 20° C. rather than at 25° C. after the implantation of corpora allata. It was also observed that the experimental animals which developed at the lower temperature retained a far larger proportion of pupal characters than in similar animals developing at 25° C.

3. *Inactivity of corpora cardiaca*

In the experiments just considered, the adult corpora allata were implanted together with the attached corpora cardiaca. However, in thirty-five additional preparations, the corpora allata were carefully dissected from the attached corpora cardiaca and then implanted into brainless diapausing pupae.

TABLE II

Tests of adult corpora allata (minus corpora cardiaca) in brainless diapausing pupae*

Adult donors	Brainless hosts	Normal development	Mixed development	No development
Cecropia	Cecropia	0	4	23
Cynthia	Cynthia	0	0	2
Cynthia	Cecropia	0	1	3
Polyphemus	Polyphemus	0	1	0
Polyphemus	Cecropia	0	0	1
Totals		0	6	29

* One to three pairs of corpora allata from male or female moths were implanted into each brainless pupa.

The results, recorded in Table II, were substantially the same as those observed in the previous experiments. Once again, a certain low percentage of brainless animals terminated diapause and transformed into pupal-adult monstrosities.

The inactivity of implanted corpora cardiaca was further confirmed in fourteen experiments in which adult corpora cardiaca were freed from corpora allata and tested, as such, in brainless diapausing pupae. No developmental response was obtained even when as many as ten pairs of adult corpora cardiaca were implanted. Indeed, in the course of twelve years of experimentation, we have never detected any trace of developmental response after the implantation of corpora cardiaca of larvae, pupae, or adults.

For present purposes it is necessary to conclude that the developmental reactions under consideration are attributable to the adult corpora allata *per se*. This implies that in a certain proportion of individuals the adult corpora allata have two effects: they first promote the initiation of adult development; they then prevent the transformation of the pupa into a normal adult moth.

4. *Effects of brain implantation*

As noted in Tables I and II, the vast majority of brainless Cecropia pupae continued to diapause when implanted with adult corpora allata. In all of these preparations the implants gave the impression of being inert. The true state of affairs is suggested by the following experiment:

Two pairs of adult corpora allata were implanted into each of five brainless Cecropia pupae. Six weeks later the pupae showed no change from their condition at the outset. Two brains of previously chilled Cecropia pupae were implanted at this time to cause the initiation of development. The latter gave rise to creatures showing large areas of pupal cuticle. In effect, the initiation of development unmasked the endocrine activity of the previously implanted corpora allata. Further information was provided by the following experiment:

Two pairs of adult Cecropia corpora allata were implanted under a facial window in each of two brainless diapausing Cecropia pupae. One month later the implants were removed and the pupae caused to develop by the injection of 125 μg. of a

purified extract of prothoracic gland hormone (ecdysone).[2] Both individuals transformed into moths which retained large areas of pupal cuticle.

This experiment shows that the presence of the brain is not necessary for the secretion of juvenile hormone by adult corpora allata. In the absence of the initiation of adult development, the implants had built up a substantial titer of juvenile hormone. But the host could not signal this fact until its development was brought about by ecdysone.

5. *Experiments on isolated pupal abdomens*

Eight abdomens were isolated from diapausing Cecropia pupae. Preparations of this type remain in permanent diapause unless provided with ecdysone by injection (Williams, 1954), or by the implantation of active prothoracic glands, or by the implantation of inactive prothoracic glands plus active brains (Williams, 1952a). In the present experiment efforts were made to evoke a developmental response of isolated abdomens by the implantation of adult corpora allata—either alone, or in conjunction with brains, prothoracic glands, or injections of ecdysone.

TABLE III

Effects of implantations into isolated abdomens of diapausing cecropia

Abdomen no.	Implant	Result
1415	1 pr. adult C.C. + C.A.	No development
1447	1 pr. adult C.C. + C.A.	No development
2123	3 pr. adult C.C. + C.A.	No development
2090..	5 pr. adult C.C. + C.A.	No development
2212	2½ pr. adult C.C. + C.A. plus 2 chilled pupal brains	No development
1515	1 pr. adult C.C. + C.A. plus 2 pr. prothoracic glands of diapausing pupae	No development
2109	3½ pr. adult C.C. + C.A. plus 4 pr. prothoracic glands of diapausing pupae	Molted to form second pupal abdomen
9320	2 pr. adult C.A. (−C.C.) plus 25 μg. of crystalline ecdysone	Molted to form second pupal abdomen

Table III summarizes the several types of preparations. It is of particular interest and importance to note that no development took place when the abdomens received only adult corpora allata. We have checked this finding in twelve additional experiments performed on isolated Cynthia abdomens; in this case the pupal abdomens were distributed at 15, 20, and 25° C. after the implantation of two to five pairs of corpora allata derived from adult Cecropia or Polyphemus. In short, no trace of development was ever observed in response to the implantation of adult corpora allata *per se*. The same negative result was also recorded in an experiment where adult corpora allata were implanted along with active brains.

The preparation numbered 9320 in Table III is of particular interest. Here, two pairs of adult corpora allata were implanted into an isolated abdomen. A

[2] I am indebted to Dr. Peter Karlson for supplying highly purified preparations of ecdysone.

month later 25 μg. of crystalline ecdysone were injected. Development began within two days. Within the following ten days the pupal abdomen transformed and molted into a second pupal abdomen (see Figure 5). This result was duplicated in two additional experiments utilizing Cynthia abdomens. It is clear that ecdysone is the prime-mover in the developmental response and that the juvenile hormone is inactive in the absence of ecdysone.

Attention is now directed to preparation 2109 in Table III. This pupal abdomen received implants of adult corpora allata plus diapausing pupal prothoracic glands. Precisely the same result was observed as after the injection of ecdysone: the pupal abdomen molted and transformed into a second pupal abdomen. In this case it seems necessary to conclude that the corpora allata activated the diapausing prothoracic glands—that, in this sense, a hormone from the corpora allata had substituted for the brain hormone. However, there is no indication in Table III that this corpus allatum hormone can substitute for ecdysone itself.

6. *Tests of adult corpora allata in previously chilled pupae*

The results considered to this point lead to the prediction that adult corpora allata should be uniformly active when tested in previously chilled pupae just prior to the initiation of adult development.

During the past ten years this prediction has been confirmed on a large scale. The experimental series includes ninety-eight preparations in which corpora allata of male and female moths of Cecropia, Polyphemus, and Cynthia were tested in chilled pupae of each of the same three species. All except eight animals gave rise to adults retaining pupal characters. In the eight negative tests the implanted glands had been derived from elderly adults just prior to death.

There was a rough correlation between the number of implanted glands and the degree to which pupal characters were preserved—a finding which will be considered in further detail in the following paper. Moreover, as was true in the earlier experiments on brainless pupae, the effects of the implanted corpora allata were amplified when the host pupae were placed at 15 or 20° C., rather than at 25° C., immediately after the implantation.

The retention of pupal characters was extreme in many of the test animals. As shown in Figure 2, the pupa transformed into a second pupa which showed only traces of adult characteristics. In several experiments performed on Polyphemus and Cecropia, the secondary pupa molted into a tertiary form. In this case, the pupal characteristics were less prominent after the second molt than after the first.

None of these animals was viable for any prolonged period after transforming into mixed forms. Although the old pupal cuticle became thin and crisp and the ecdysial lines were eroded to the surface, spontaneous escape from the old pupal cuticle occurred only in individuals showing minimal retention of pupal characteristics. All other animals remained enveloped in the old pupal cuticle until they died or were sacrificed.

In many of the individuals the molting process proceeded to a normal terminal phase accompanied by a complete breakdown of the old endocuticle and a partial or complete resorption of the molting fluid. Yet, for some unexplained reason, the insect failed to undertake the vigorous muscular efforts that accompany a normal ecdysis. It did not "try to molt" even though it possessed the nervous and

muscular equipment to do so. The use of forceps was therefore necessary to peel off the old pupal exuviae.

In many individuals it was difficult or impossible to withdraw the lining of the old tracheal tubes through the spiracular openings. Indeed, in the case of Cecropia, the larger branches of this old system became stiff and melanized and therefore incapable of being shed. The net effect is that the juvenile hormone is a lethal agent for all these Saturniid pupae.

7. *Inactivity of killed corpora allata*

The high activity recorded for implanted adult corpora allata suggested the possibility that substantial amounts of hormone might be stored within the glands themselves. This prospect was tested in five experiments. In one experiment eight adult Cecropia corpora allata were frozen and thawed twice at −40° C. and then implanted into a previously chilled pupa. Normal development ensued.

In four other experiments adult corpora allata, in numbers ranging from 9 to 44, were homogenized in 0.1 ml. of insect Ringer and then introduced into four previously chilled pupae. All four animals developed into normal adult moths. Evidently, little or no hormone is stored in the living gland, for the activity of a single living adult corpus allatum was not duplicated by the implantation of up to forty-four dead glands.

Discussion

1. *Secretion of the juvenile hormone by the adult corpora allata*

The experimental results demonstrate the endocrine activity of the corpora allata of Cecropia, Polyphemus, and Cynthia moths. As is amply evident in Wigglesworth's (1954) recent review, this finding is consistent with the picture presented in all other insects that have been studied in detail including several families of Lepidoptera. In the Cecropia silkworm the corpora allata, when removed and tested, are found to be more active in the adult moth than at any other stage in the life history (Williams, unpublished data). Moreover, there is general agreement that at least one of the secretory products of the adult corpora allata is the same juvenile hormone which is secreted weeks or months earlier by the corpora allata of the immature insect. This conclusion was first proposed by Pflugfelder (1938a, 1938b) and Pfeiffer (1945), and will be further documented in the subsequent papers in this series.

2. *The role of the juvenile hormone in adult moths*

We have been unable to detect any function for the corpora allata in the pupal or adult stages of these Lepidoptera. Thus, as we have seen, the corpora allata can be removed from pupae of either sex without disturbing the development of normal, viable, sexually mature moths. These findings are the same as those reported for *Bombyx mori* by Bounhiol (1938) and Fukuda (1944). The present study enlarges the negative evidence by showing that the absence of corpora allata fails to interfere with the maturation of functional gametes and the production of normal offspring.

The situation in the Lepidoptera therefore departs from that described for most other orders of insects where the corpora allata are necessary for the deposition of yolk in the adult female and for the secretory activity of the accessory glands in the adult male (for summary, see Wigglesworth, 1954, pages 77–80). In the Lepidoptera which have been studied, all these functions can go forward in the absence of corpora allata. For the sexual maturation of both males and females all that is required is the presence of prothoracic gland hormone (ecdysone). The brain hormone is also unnecessary for the sexual maturation of these silkworms. Pupae from which the brain, corpora cardiaca, and corpora allata have been removed develop into sexually mature moths after the injection of crystalline ecdysone (Williams, 1954).

Adult Lepidoptera therefore present the paradoxical picture of the presence of highly active corpora allata for which there appears to be no apparent function. However, it is worth recalling that corpora allata have been tested only in species of adult Lepidoptera which are short-lived and unable to feed. In adults of the giant silkworms, as in the commercial silkworm, functional mouth-parts are absent. Consequently, the duration of the adult stage is greatly curtailed: ripe eggs must be ready for oviposition at the time of adult emergence. In short, the absence of mouth-parts has enforced on these short-lived moths a precocious maturation of the gonads during the course of pupal-adult development. Indeed, months before the development of the adult moth, the proteins which later appear in the yolk of the eggs are already present in high concentrations in the blood of the diapausing pupa (Telfer, 1954).

It is among the feeding, long-lived species of adult Lepidoptera that one would anticipate a gonadotropic function for the corpora allata akin to that seen in most other orders of insects. This inference is in accord with the histological studies of Kaiser (1949) on long-lived butterflies of the genus *Vanessa*. Presumably, in the Ephemeroptera and other non-feeding adults one should find the same picture as presented by the Saturniidae.

The absence of functional adult mouth-parts is clearly a secondary affair in the evolution of the Lepidoptera. Indeed, the very same moths contain digestive tracts of normal organization, but of no apparent function. Evidently, the presence of active corpora allata is a memento of a more primitive endocrinological situation.

3. *Biological role of the juvenile hormone*

The juvenile hormone plays no role in the transformation of a pupa into an adult moth. All that is required is that the juvenile hormone be absent throughout the early phases of this transformation (Williams, 1952b). This conclusion is in line with the finding that the corpora allata are inactive throughout the entire pupal stage and during the first two-thirds of adult development (Williams, unpublished data).

A pupa can be supplied with juvenile hormone by the implantation of living, active corpora allata obtained from larvae or adults. However, as demonstrated in the experiments on isolated pupal abdomens (Table III), the juvenile hormone has no effects in the absence of the prothoracic gland hormone, ecdysone. Only when the abdomen is provided with this hormone can one detect any action of

the implanted corpora allata. The outcome is that the pupal abdomen terminates diapause, molts, and transforms into a second pupal abdomen (Fig. 5).

Substantially the same result is seen in experiments performed on brainless diapausing pupae. Here again the implantation of adult corpora allata is inconsequential unless ecdysone is supplied by injection or by the secretory activity of the animal's own prothoracic glands. The juvenile hormone then opposes the transformation of the pupa into an adult moth. The result (Figs. 3 and 4) is a creature showing to varying degrees a retention of pupal characters of the type previously described by Piepho (1952) and Williams (1952b). When the titer of juvenile hormone is high, then one may witness the formation of a *bona fide* second pupal instar—a phenomenon hitherto unknown in any insect (Fig. 2). But, even in the presence of the highest concentrations of juvenile hormone, we have never observed in this material the reappearance of larval characters such as described in *Rhodnius* (Wigglesworth, 1954, 1957, 1958).

4. *Mimicking of brain hormone*

In a certain proportion of brainless diapausing pupae the implantation of active corpora allata causes the termination of diapause and the initiation of adult development. This result is not seen in isolated pupal abdomens or other preparations lacking prothoracic glands. But, as noted in Table III, the developmental reaction becomes possible if an isolated abdomen receives active corpora allata plus inactive prothoracic glands, or active corpora allata plus an injection of ecdysone (Fig. 5). Moreover, in numerous experiments to be described on a later occasion, the development of brainless diapausing pupae has been provoked by the injection of crude or purified extracts of juvenile hormone. Evidently, under certain undefined conditions, a hormonal secretion of the corpora allata can activate the prothoracic glands and, in this sense, mimic the function of the brain hormone. Whether this hormone is the juvenile hormone or some further secretory product of the corpora allata is impossible to state at the present time. A decision on this point will become possible only when the juvenile hormone is isolated and tested in pure form.

The finding that the corpora allata can turn on the prothoracic glands has an obvious bearing on the endocrine control of larval molting. If the corpora allata can activate the pupal prothoracic glands, there is no reason to suppose that they cannot do so in the immature larva.

We begin to see a multiplicity of agencies which can promote the secretion of ecdysone by the prothoracic glands. The brain can turn on the prothoracic glands. Ecdysone can turn on the prothoracic glands (Williams, 1952a, 1954). And, evidently, under certain undefined conditions, so also can the corpora allata. Nature has apparently found it prudent to surround the prothoracic glands by a net-work of controls. The present study suggests that the corpora allata are a part of that net-work.

Summary

1. Juvenile hormone is secreted in high concentration by the corpora allata of the adult Cecropia moth.

2. Notwithstanding this fact, the juvenile hormone has no apparent function in the adult moth. Extirpation of the corpora allata in the pupal stage fails to interfere with the production of normal moths whose gametes give rise to normal offspring.

3. The corpora allata are inactive during the entire pupal stage as well as during the first two-thirds of adult development. If active corpora allata are implanted into a pupa just prior to the initiation of adult development, the juvenile hormone acts to oppose the differentiation of the adult moth. Development gives rise to an insect showing a mixture of pupal and adult characters. In the presence of high concentrations of juvenile hormone the pupa molts and transforms into a second pupa showing only traces of adult characters.

4. The biological action of juvenile hormone is seen only in the presence of active prothoracic glands or their secretory product, ecdysone. Isolated pupal abdomens fail to respond to juvenile hormone unless ecdysone is simultaneously present. When both hormones are present, the pupal abdomen terminates diapause, molts, and transforms into a second pupal abdomen.

5. Evidence is presented that the corpora allata secrete a factor which can mimic the brain hormone and activate the prothoracic glands. This finding is considered in relation to the endocrine control of larval molting.

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